The Potential of Dynamic Controlled Atmospheres and Possible Mechanisms in Mitigating Superficial Scald in Apples cv. 'Granny Smith'

By

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DECLARATION

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

The development of a postharvest method for controlling superficial scald, maintaining quality and reducing postharvest losses of 'Granny Smith' apples is essential in maintaining the competitiveness of the South African apple industry. Previously, the South African apple industry relied on diphenylamine (DPA) for controlling scald disorder; however, increasing consumer concerns and reductions in maximum residue levels (MRLs) have highlighted the urgent need for alternative control strategies. Currently, there is no effective non-chemical method for controlling superficial scald for South African apple producers. The overall aims of this study were (a) to examine the potential of dynamic controlled atmospheres (DCA) in controlling superficial scald in apples, and (b) to investigate the mechanism of action of DCA in controlling scald, should it be effective.

To get a deeper understanding of superficial scald etiology and physiological dynamics of apples, studies in paper 2 and 3 were conducted. In paper 2, studies on antioxidants contents and phytochemical properties of apples harvested at pre-optimal and optimal maturity were conducted. Significant increases in fruit antioxidant capacity and ascorbic acid concentration occurred with increasing maturity. Fruit harvested at optimal maturity had lower total phenolic contents compared to pre-optimal maturity. Phenolic compounds including catechin and quercetin were also higher in pre-optimal compared to optimal maturity. In paper 3, an attempt was made to classify apples with different levels of scald severity based on metabolomics analysis. The results showed that ethylene, α-farnesene, 6-methyl-5-hepten-2-one (MHO) and reactive oxygen species (ROS) increased with scald severity but declined in severely scalded fruit. Discriminant analysis successfully classified fruit based on scald severity. Ethylene, ROS and lipid peroxidation were identified as the major contributors in separating the five scald severity levels studied.

Studies in paper 4 focused on whether DCA is effective in controlling superficial scald. The minimum period for the exposure of fruit to DCA before an extended shipment period of 10 weeks was also investigated. The results showed that DCA was highly effective in controlling scald for both pre-optimal and optimal harvested fruit. The results further demonstrated that DCA stored fruit can be shipped for 6 weeks; however, extending the shipping period up to 10 weeks might lead scald development and undesired fruit quality. Fruit stored in DCA before shipment generally had higher flesh firmness and ground colour. It was also shown that DCA inhibit scald by retarding the accumulation of scald-associated

metabolites such as α-farnesene and MHO. Paper 5 focused on the impact of DCA on ROS, antioxidant capacity and phytochemical properties of stored apples. Using principal component analysis, two clusters which could be identified as DCA and RA stored fruit were noticed. Compared to RA stored fruit associated with higher ROS and lipid peroxidation, fruit stored at DCA was characterized by higher contents of ascorbic acid, total phenolics and antioxidant pool.

The research reported in paper 6 investigated the efficacy of repeated application of DCA on apples with high scald potential. During the marketing season, an unexpected demand of fruit often leads to the opening and resealing of storage chambers. Thus, the efficacy of a repeated DCA treatment after an interruption period at RA was investigated. Fruit were stored for up to 16 weeks in DCA with a 14 d interruption in RA at -0.5 °C, 95% RH. The results showed that efficacy of DCA was not significantly affected by the interruption. However, the development of 1% scald after 4 months of storage could be an economic setback for fruit producers. In paper 7, the influence of DCA on aroma volatiles was assessed. DCA stored fruit had significantly lower total amount of volatiles detected compared to fruit stored in RA. Notably, the production of 1-butanol, 1-hexanol and 1-hexen-ol by fruit stored in DCA were only 42%, 38% and 39%, respectively, of the amounts detected in the RA. The known characteristic flavour of 'Granny Smith' apples was attributed to the production of ethyl-2-methylbutyrate, ethyl hexanoate and hexyl acetate. The contribution of these three aroma volatiles was higher with increasing storage duration.

In paper 8, the research identified effective variables that could be used to develop prediction models for superficial scald incidence in harvested 'Granny Smith' apples. Stepwise multiple regression found MHO, antioxidant capacity (FRAP), ascorbic acid and lipid peroxidation to be the best combination of predictive variables for scald. After validation, this combination gave a good prediction of scald incidence (R^2 = 0.94). The identified variables proved to be effective regardless of fruit maturity status. The results from this thesis provide an alternative non-chemical postharvest technology for the South African apple industry. The study further provides insights on the mechanism of action of DCA in controlling scald and maintaining fruit postharvest quality of 'Granny Smith' apples. Overall, the results contained in this thesis will be very instrumental in future optimisation of DCA technology in the apple industry, and provides a valuable guide for improved the storage of apples susceptible to superficial scald.

OPSOMMING

Die ontwikkeling van ’n na-oes metode vir die beheer van oppervlakkige brandvlek, die behoud van gehalte en die vermindering van na-oes verliese van die ‘Granny Smith’ appel is belangrik as Suid-Afrika kompeterend wil bly in die appelindustrie. In die verlede het die Suid-Afrikaanse appelindustrie difenielamien (DPA) benut vir die beheer van oppervlakkige brandvlek maar die groeiende bekommernis van verbruikers en die vermindering in maksimum residuvlakke het die behoefte aan alternatiewe beheerstrategieë beklemtoon. Daar is tans geen alternatiewe nie-chemiese metodes wat Suid-Afrikaanse appelboere kan gebruik om oppervlakkige brandvlek te beheer nie**.** Die doel met hierdie navorsing is (a) om die potensiaal van dinamies beheerde atmosfeer (DBA) en die beheer van oppervlakkige brandvlek in appels te ondersoek, en (b) om die meganisme van aksie van DBA te ondersoek, indien dit wel effektief bevind word in die beheer van oppervlakkige brandvlek in appels.

Die navorsing wat in Artikels 2 en 3 opgeteken is, is gedoen om ’n dieper begrip van oppervlakkige brandvlek etiologie en die fisiologiese dinamika van appels te bekom. In Artikel 2 is die bevindings oor die chemiese kenmerke van appels wat geoes is by pre-optimale en optimale oesrypehied, opgeteken. Betekenisvolle vermederinge in die vrugte se antiodatiewe status en askorbiensuur konsentrasie vind met volwassenheid plaas. Vrugte wat by optimale rypheid geoes word het ’n laer totale fenoliese inhoud vergeleke met vrugte wat by pre-optimale volwassenheid gepluk word. Fenoliese samestellings insluitende catechin en quercetin is ook hoër by volwasse vrugte. In die navorsing wat in Artikel 3 opgeteken is, is daar gepoog om appels met verskillende vlakke van brandvlek deur middel van metabolomiese analise te klassifiseer. Die resultate toon dat etileen, α-farnesene, 6-metiel-5-hepten-2-een (MHO) en die reaktiewe suurstof spesie (RSS) toeneem hoe erger die brandvlek raak maar afneem in erg gebrandvlekde vrugte. Die vrugte is suksesvol geklassifiseer volgens hoe erg die brandvlek voorgekom het deur middel van onderskeidende ontledings. Etileen, RSS en lipied peroksidasie is identifiseer as die hoof bydraers tot die onderskeiding van die vyf brandvlek vlakke wat bestudeer is.

In die studie wat in Artikel 4 opgeteken is, is die fokus op of dinamiese beheeranaliese, oppervlakkige brandvlek doeltreffend kan beheer. Die minimum periode vir die blootstelling van vrugte aan dinamies beheerde atmosfeer voor ’n uitgebreide verskepings periode van 10 weke is ook ondersoek. Die resultate toon dat dinamies beheerde atmosfeer hoogs effektief is in die beheer van brandvlek beide in vrugte wat voor die optimale tyd of op

die optimale tyd geoes is. Die resultate het ook getoon dat vrugte wat in ’n dinamies beheerde atmosfeer gestoor is, vir ses weke verskeep kan word; maar dat as die periode tot tien weke verleng word, brandvlek kan ontwikkel en die gehalte van die vrugte kan afneem. Vrugte wat voor verskeping in DBA gestoor is, is fermer en die agtergrondkleur is beter behou. Daar is ook getoon dat DBA brandvlek ontmoedig deur om die akkumulasie van metaboliete wat met brandvlek geassosieer word, soos α-farnesene en MHO te verminder. In Artikel 5 is die fokus op die impak van DBA op RSS, antioksidant vermoë en die fitochemiese kenmerke van gestoorde appels. Deur om die hoofkomponente te ontleed is twee groepe wat as DBA en verkoelde lug (RA) gestoorde vrugte identifiseer is, uitgeken. In vergelyking met vrugte wat onder RA toedtande gestoor is, en wat geassosieer was met hoë ROS en lipied peroksidasie, is vrugte wat in DBA gestoor is, gekenmerk deur ’n hoër askorbiensuur inhoud, totale fenoloë en oksidante.

In Artikel 6 is die bevindings van ’n ondersoek na die doeltreffendheid van herhaaldelike toepassing van DBA op appels met ’n hoë brandvlek potensiaal, opgeteken. Dit gebeur dikwels dat daar gedurende die markseisoen ’n onverwagte vraag na vrugte ontstaan en dat die stoorkamers dan oopgemaak en weer verseël word. Dus is die doeltreffendheid van herhaaldelike DBA behandeling na ’n periode van RA ondersoek. Vrugte is vir tot 16 weke in DBA gestoor met ’n onderbreking van 14 dae in RA teen -0.5 °C, 95% RH**.** Die bevindinge het bewys dat die doeltreffendheid van DBA nie merkbaar deur die onderbreking aangetas is nie. Die ontwikkeling van 1% brandvlek na 4 maande in die stoorkamers mag egter deur die produsente as negatief beskou word. In Artikel 7, word die invloed van DBA op die aromtiesevlugtige stowwe geassesseer. Vrugte wat in DBA gestoor is, het ’n merkbaar laer totale hoeveelheid aromatiese vlugtige stowwe getoon, in vergelyking met vrugte wat in ’n RA gestoor is. Die produksie van 1-butanol, 1-heksanol en 1-heksen-ol in vrugte wat in DBA gestoor is, is egter net 42%, 38% en 39%, onderskeidelik van die wat in RA gestoor is. Die kenmerkende geur van ‘Granny Smith’ appels is die gevolg van die produksie van etiel-2-metielbutyraat, etiel heksanoate and heksiel asetaat.Die bydrae van hierdie drie aromatiese vlugtige stowwe was hoër na’n uitgebreide stoortydperk.

In Artikel 8 word die navorsing beskryf wat doeltreffende veranderlikes identifiseer wat gebruik kan word om voorspellingsmodelle vir die hoeveelheid van oppervlakkige brandvlek in geoesde ‘Granny appels’ te ontwikkel. Deur stapsgewyse veelregressie is daar gevind dat MHO, antioksidant vermoë, askorbiensuur en lipiede peroksidasie die beste kombinasie is vir die voorspellende veranderlikes vir brandvlek. Nadat dit geldig gevind is,

het hierdie kombinasie ’n goeie voorspelling vir die voorkoms van brandvlek (R^2 = 0.94) verskaf. Die geïdentifiseerde veranderlikes was effektief vir alle vrugte, sonder inagneming van volwassenheid. Die bevindinge van hierdie navorsing verskaf ’n alternatiewe nie-chemiese na-oes tegnologie vir die Suid-Afrikaanse appelindustrie. Verder is nuwe insigte bekom in die meganismes van aksie van DBA wat betref die beheer van brandvlek en die behoud van na-oes gehalte van ‘Granny Smith’ appels. In die geheel sal die bevindinge van hierdie tesis bydra tot die toekomstige optimalisering van DBA tegnologie in die appelindustrie, en waardevolle riglyne verskaf word vir die verbetering van die stoor van appels wat vatbaar is vir oppervlakkige brandvlek.

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TABLE OF CONTENTS

This thesis is a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable. Language and styles used in this thesis are in accordance with the requirements of the Postharvest Biology and Technology.

LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications

- Mditshwa, A., Vries, F., van der Merwe, K., Crouch, E., Opara, U.L., 2015. Antioxidant contents and phytochemical properties of apples (cv. Granny Smith) at different harvest times. *South African Journal of Plant and Soils*, DIO:10.1080/02571862.2015.1028489

Conference presentations

- Mditshwa, A., Vries, F., van der Merwe, K., Crouch, E., Opara, U.L., 2015. The role of α-farnesene and 6-methyl-5-hepten-2-one in superficial scald development. *Student Symposium in Analytical Sciences*, Stellenbosch, South Africa, 27 March 2014.

- Mditshwa, A., Opara, U.L., Crouch, E., Vries, F., van der Merwe, K., 2015. Recent developments in controlling superficial scald in apples. Hortgro[science] *Postharvest Seminar*, Stellenbosch, South Africa, 22 January 2015.10.11

GENERAL INTRODUCTION

1. Background

Apple (*Malus domestica*) is one of the most frequently consumed fruit globally and in South Africa (PPECB, 2012). Apples contain antioxidants and phytochemicals such as chlorogenic acid, quercetin, phloridzin, procyanidin and catechin (Hammerstone et al., 2000; Boyer and Liu, 2004). In fact, epidemiological studies have correlated high apple consumption to reduced risk of asthma, diabetes, certain cancers and cardiovascular diseases (Liu, 2003; Boyer and Liu, 2004; Liu et al., 2005; Adyanthaya et al., 2010). It is widely cultivated and South Africa is amongst the top twenty producing countries with 760 936 tonnes/year (PPECB, 2012). Generally, the supply of apple fruit is often higher than the demand. To avoid postharvest losses and increase marketability in a window period where demand is higher than supply, fruit is cold stored for several months. 'Granny Smith' apple, one of the most important export cultivars, is susceptible to superficial scald after long-term storage, which manifests as brown patches on fruit surface (Jemric *et al*., 2006; Sabban-Amin *et al*., 2011).

Scalding is associated with accumulation of α-farnesene in the fruit peel (Isidoro and Almeida, 2006), while the symptom is highly correlated with 6-methyl-5-hepten-2-one (MHO), an end-product of α-farnesene oxidation. The accumulation of α-farnesene is also linked to increased ethylene production (Zanella, 2003). The role of fruit antioxidant status on scalding is not known. The synthetic antioxidant diphenylamine (DPA) is effective in controlling scald (Sabban-Amin *et al*., 2011); however, the EU Commission has reduced the maximum residue level (MRL) for non-approved active substances for which consumer concerns have been identified, including DPA (APAL, 2013). The reduction of MRL and potential ban of DPA poses considerable economic risk to the South African pome fruit industry. New and innovative strategies are therefore needed to control superficial scald.

With the reduction of DPA MRL from 5 to 0.1 ppm (effective from December 2013), an alternative is urgently required. Preliminary research findings by the South African Agricultural Research Council-Infruitec/Nietvoorbij and others (Zanella *et al*., 2008) have highlighted the potential of dynamic controlled atmosphere (DCA) storage as an alternative

non-chemical treatment; however, array of questions are yet unanswered. Pre-harvest factors such as climatic conditions and maturity affect scald incidence (Ahn *et al*., 2007). The efficacy of DCA on fruit harvested at pre-optimal and optimal maturity is not known. Furthermore, the influence of seasonal variations on DCA effectiveness to control superficial scald is not yet known. To date, the mode of action of DCA in inhibiting scald is not well understood.

2. Aims and objectives

2.1. Aims

The overall aims of this research were to examine the potential of dynamic controlled atmospheres in controlling superficial scald in apples, and to further investigate the mechanism of action.

2.2. Objectives

The specific objectives of this study were to:

a) Assess effects of DCA on scald and biochemical precursors in fruit at different maturities;

(b) Determine critical application period for DCA to inhibit scald; and

(c) Investigate the influence of intermittent breaks on DCA effectiveness

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PAPER 1

Superficial Scald in Apples: Biochemistry, Physiology, Control and Modelling - A Review

Abstract

Superficial scald is an important physiological disorder causing brown or black patches on the exocarp after long-term cold storage of susceptible apple and pear cultivars. Currently, the control of this disorder is achieved through the application of ethylene inhibitors such as 1-methylcyclopropene (1-MCP) and antioxidants such as diphenylamine (DPA). Non-chemical methods such as initial low oxygen stress (ILOS), controlled atmosphere (CA) and dynamic controlled atmosphere (DCA) have demonstrated potential to control the disorder. This review paper highlights the progress made in understanding and controlling superficial scald. Recent research on the use of non-chemical methods for controlling scald is discussed. In addition, research efforts focused on applying mathematical modelling for predicting superficial scald are reviewed. Moreover, the feasibility of using non-destructive methods for quantifying scald-related metabolites is examined and prospects for future research are highlighted.

Keywords: Superficial scald, apple, postharvest, α-farnesene, conjugated trienols, 6-methyl-5-hepten-2-one

1. Introduction

Superficial scald is a physiological disorder of apples [*Malus domestica*] and pears [*Pyrus communis* L. and *Pyrus serotina* Redh.] that causes quality loss following long-term cold storage (Figure. 1). The name ‘superficial scald’ is associated with the disruption of tissues immediately beneath the epidermis of the fruit, with tissue browning not extending to the mesocarp of pulp. Array of factors such as cultivar, seasonality, growing location, pre-harvest temperature, fruit maturity, and cold storage duration have been implicated in scald development (Ahn et al., 2007; Emongor et al., 1994; Rao et al., 1998; Watkins et al., 2000a; Lurie and Watkins et al., 2012).

Alpha-farnesene accumulation in fruit peel (cuticle, epidermis, and hypodermis) during cold storage, combined with the oxidation of conjugated trienols (CTols) is arguably the major cause of superficial scald (Isidoro and Almeida, 2006). The disruption of cell membrane by CTols causes polyphenoloxidase-mediated browning of fruit peel with hypodermal cell layers developing necrosis. In fact, Fidler (1950) reported browning of epidermal and hypodermal cell in early development of scald. Moreover, the intensity of hypodermal cells browning increased with scald severity, leading to death of cells in severely injured cells. An array of postharvest techniques is used to combat superficial scald. Postharvest treatments such as intermittent warming, hot water dips, and use of ethylene inhibitors such as 1-MCP and antioxidants such as diphenylamine (DPA) has been very effective.

Table 1 summarises several reviews which examined various aspects of superficial scald in both apples and pears including biological, physiological and biochemical principles. Several methods for combating scald development have also been reviewed. Ingle (2001) reviewed the influence of both pre-harvest and postharvest factors influencing superficial scald. A recent critical and informative review by Lurie and Watkins (2012) on the etiology and control of superficial scald has led to better understanding of this disorder. None of these reviews examined the potential of dynamic controlled atmosphere (DCA) storage, and predictive physiology and biochemistry towards achieving optimal benefits of currently used technologies for inhibiting superficial scald. This paper reviews the developments in superficial scald research, with emphasis on intrinsic and extrinsic factors. Different postharvest methods and recently identified non-chemical technologies for controlling scald are also reviewed. Future prospects of superficial scald research are also identified.

2. Biochemistry and physiology of superficial scald

Superficial scald physiology is significantly influenced by the intrinsic properties of the produce, as well as extrinsic factors as summarized in Table 2. Both pears and apples vary in their intrinsic properties. For instance, scald resistant ‘Golden Delicious’ apples has a high phenolic concentration, whereas scald susceptible cultivars such as ‘Cortland’ and ‘Empire’ have lower phenolics concentrations (Ju and Bramlage, 1999).

2.1 Intrinsic factors influencing superficial scald development in apples

Various intrinsic factors affecting scald development have been reported. However, scald biochemistry studies have focused on α-farnesene and its oxidation products such as 6-methyl-5-hepten-2-one (MHO) and conjugated trienols (CTols), factors such as antioxidant content and membrane lipids have received little attention. A pioneering laboratory effort by Anet (1974) revealed that antioxidants could play a critical role in inhibiting scald development. According to Lurie and Watkins (2012), no single postharvest method for controlling scald is suitable for different pome fruit industries around the globe. Therefore, an overall understanding of the intrinsic factors involved in scald development is very critical for developing postharvest technologies. Cultivar, fruit maturity, gene expression, ethylene production and membrane lipids are some of the significant factors involved in scald development.

2.1.1 Cultivar

The scald susceptibility of various cultivars varies. Generally, some cultivars are highly susceptible whilst some are resistant (Table 3). For instance, cultivars such as ‘White Angel’, ‘Idared’, ‘Gala’, and ‘Golden Delicious’ are scald resistant (Fernández-Trujillo et al., 2003; Rao et al., 1998), while scald is more prevalent on ‘Rome Beauty’, ‘Law Rome’, ‘Cortland’, ‘McIntosh’ and ‘Granny Smith’ (Fernández-Trujillo et al., 2003; Rao et al, 1998). Previous studies predominantly focused on postharvest treatments rather than developing resistant cultivars through genetic manipulation. This is regardless of well researched and articulated introductory information of genetic involvement on scald etiology. Globally, a scald susceptible ‘Granny Smith’ is the popular cultivar; however, studies on development of scald resistant ‘Granny Smith’ have not yet been initiated. This is despite having scald resistant ‘Golden Delicious’ DNA sequenced. There are probably other genes yet to be identified that could play an imperative role in developing scald resistant cultivars.

2.1.2 Fruit maturity

Fruit maturity is another intrinsic factor that influences postharvest potential and flavour development in apples (Echeverria et al., 2004). Based on harvesting time, maturity can be categorized into three groups, namely; early, optimal and late maturity. Superficial scald is more

prevalent in pre-optimally than optimally harvested fruit (Lurie and Watkins, 2012; Wang and Dilley, 1999). In fact, scald severity decreases with advancing fruit maturity. For example, scald incidence in early and late harvested 'Granny Smith' apples was 85.4% and 24.4%, respectively when stored at 0 °C for 8 months (Erkan and Perkmezci, 2004). Although scald is less prevalent on late harvested fruit, both early and late harvests have major drawbacks on fruit quality. Reduced fruit firmness is generic on later harvested fruit whilst impaired flavour development is prominent on earlier harvested fruit. Desirable characteristics such as prolonged ripening period and firmness retention are common in unripe fruit (Echeverria et al., 2004). Alpha-farnesene concentration can be higher at pre-optimal stage, increasing with ethylene production and subsequently superficial scald (Emongor et al., 1994). On the other hand, advancing fruit maturity is coupled with higher ethylene production and lower starch and chlorophyll content. The relationship between superficial scald and maturity stage is very complex. In addition to ethylene and α-farnesene evolution, reactive oxygen species, phytochemicals and endogenous antioxidant systems are involved. According to Rao et al. (1998) and Whitaker et al. (2000), the activity antioxidant enzymes may indeed be more important than α-farnesene concentration. Accordingly, the difference in scald susceptibility in pre-optimally and optimally harvested fruit could be linked to antioxidant activity.

2.1.3 Ethylene content

Ethylene content of the fruit is another intrinsic factor that influences scald development (Ingle, 2001). Ethylene has a fundamental role in physiological changes associated with superficial scald development; however the role is not clear. Alpha-farnesene synthesis is associated with ethylene production (Ju and Curry, 2002). Both ethylene production and α-farnesene synthesis were inhibited by aminoethoxyvinylglycine (AVG) applied pre-harvest (Ju and Curry, 2000a; Mir et al., 1999). Moreover, ethylene production and subsequently α-farnesene is reduced by 1-MCP. However, the major role of ethylene in scald physiology remains unclear. Studies by Dandekar et al. (2004) and Pesis et al. (2009) investigated the role of ethylene in storage physiological disorders of apples using genetic manipulation of ethylene biosynthesis pathway. The lines with less ethylene and α-farnesene synthesis were discovered. The identification of *AFS1*, the ethylene-dependent gene encoding α-farnesene synthate in apples

and pears is instrumental in genetic manipulation (Lurie et al., 2005; Gapper et al., 2006; Tsantili et al., 2007).

2.1.4 Volatile compounds

Volatile composition of the fruit is another intrinsic factor that significantly influences scald development in both apples and pears (Franeti et al., 2014; Shabban-Amin et al., 2011; Whitaker, 2004). The pathway of α-farnesene synthesis indicates that ethylene production plays a critical role (Whitaker, 2004). An early study by Brooks et al. (1923) found reduced scald incidence in apples and pears wrapped with mineral oil impregnated paper. It was thereafter discovered that volatile substances were absorbed by the paper. Alpha-farnesene is the common volatile found on scalded pome fruits. Generally, apple and pear cultivars susceptible to scald contain high α-farnesene content compared to resistant cultivars (Ingle, 2001). Additionally, less mature fruit accumulate high α-farnesene content and subsequently highly susceptible to scald (Huelin and Coggiola, 1968; Whitaker et al., 1997). Correlative studies have shown a close relationship between light intensity and and α-farnesene, and consequently on scald incidence. For instance, 'Granny Smith' apples exposed to high light intensity before storage at 1 °C had lower α-farnesene content and reduced scald incidence after 6 months storage (Rudell and Mattheis, 2009).

Scald symptoms are closely linked to MHO and CTs, the end-products of α-farnesene oxidation. For instance, high MHO accumulation after removal of fruit from cold storage coincides with scald symptoms in 'Cortland' (Mir et al., 1999) and 'Granny Smith' apples (Wang and Dilley, 2000a). Recently, Farneti et al. (2015a) found MHO to be significantly correlated to early symptoms of superficial scald development. Using a new version of mass spectrometer based on proton transfer reaction (PTR-ToF-MS), Busatto et al. (2014) also found MHO accumulation to coincide with development of superficial scald symptoms. On the contrary, Ju and Curry (2002) and Rowan et al. (2001) reported no scald incidence in MHO treated fruit. It would be reasonable to hypothesize that antioxidant activity of stored apples may be more important than MHO concentration. Additionally, MHO production, as opposed to its presence, could be more critical to understanding the cause of scald development (Ju and Curry, 2002; Rowan et al., 2001).

CTs are volatile compounds implicated in scald development. CTs are composed of at least two conjugated hydrocarbons and hydroperoxide, and are products of α-farnesene oxidation (Ingle, 2001). A study by Rowan et al. (1995), established that the main CT hydrocarbon, now called 2,6,10-trimethydodeca-2,7(E), 9(E),11-tetraen-6-ol constitutes 89-95% while hydroperoxide only contributes 5-11% to the whole body of CTols. Although CTs have been reported to influence scald susceptibility, there are inconsistences on these findings. For instance, CTs are associated to scald development in 'Granny Smith' apples (Huelin and Coggiola, 1970; Moggia et al., 2010). A recent study by Farneti et al. (2015b) showed that CTs exist in much higher levels in scalded 'Granny Smith' and 'Cripps' Pink' apples. On the contrary however, Ingle (2001) reported that it remains speculative to relate scald to CTs, this is attributed to the fact that no breakdown products of CTs have been described. Ingle and D'Souza (1989) recorded a CT concentration of 18-51 nmole cm^{-2} on 'Delicious' apples whilst a highly scald susceptible 'Granny Smith' contained the lowest concentration of 13-16 nmole cm^{-2}. At the moment, it is clear that CTs concentration is not a reliable indicator of superficial scald development. In case of α-farnesene, changes during fruit maturation have been established and they played a significant role in developing technologies for controlling scald. The changes in CTs concentrations during fruit maturation are not significant compared to α-farnesene changes reported in literature. For instance, Barden and Bramlage (1994) working on 'Cortland' apple, recorded CT281 of 1 nmole cm^{-2} and 3 nmole cm^{-2} in September and October, respectively. Moreover, the increase in CTs during maturity stages was less than the increase during refrigerated storage. However, CTs could not be correlated with α-farnesene. In another study by Du and Bramlage (1993) on 'Cortland' and 'Delicious' apples it was found that CTs are not involved in scald development during cold storage. 'Cortland' had highest CTs concentration; however, there was no scald development. In contrast, CT concentration of 0.11 nmole cm^{-2} was reported in 'Granny Smith' apples harvested in April and 2.96 nmole cm^{-2} after 20 weeks of cold storage (Watkins et al., 1995). It is clear that research findings on the relationship volatile compounds have with scald development has not been consistent. Several methods to quantify α-farnesene, CTs and MHO have been previously used by researchers (Table 4). A spectrophotometric method at wavelength of 269 or 282 nm is commonly used since early 1970s (Huelin and Coggiola, 1970). However, the detection of other compounds at these wavelengths is a major setback. As a result, recent studies have opted to use high performance liquid

chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) with more accuracy and precision in measuring volatile compounds. Utilization of new technologies such as electronic nose could probably provide conclusive information on the role that CTs and MHO play in scald development.

2.1.5 Reactive oxygen species (ROS)

ROS play a critical role in the development of physiological disorders. ROS cause the oxidative stress that consequently results to imbalances in metabolism, high respiration rate, reduced ability of biological systems to detoxify toxic metabolites. Low storage temperatures trigger plant cells to produce ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), the by-products of electron flow disruption in the mitochondria (Pinhero et al., 1997; Purvis et al., 1995). Superficial development is closely linked to higher accumulation of ROS in the peel. For instance, Rao et al. (1998) reported higher tissue concentration of ROS in scald-susceptible 'White Angel x Rome Beauty' apple lines compared to lower concentration in scald resistant lines. Sabban-Amin et al. (2011) and Pesis et al. (2014) using confocal microscopy which visually exhibit the appearance of ROS molecules showed that ROS exist in much higher levels in scalded 'Granny Smith' apples. Recently, a strong relationship between ROS, specifically H_2O_2, and scald incidence and severity in 'Fuji' apples has been reported (Lu et al., 2014). Antioxidants and phytochemicals are the major biological systems responsible for scavenging ROS. A high antioxidant pool is generally associated with low scald incidence; moreover, reduced α-farnesene oxidation is reported (Barden and Bramlage, 1994). Ju et al. (1996) working on 'Delicious' apples, observed a strong correlation between scald visual symptoms and reduction in phenol concentration. Additionally, high phenol concentration was found on resistant 'Golden Delicious' than scald susceptible cultivars, 'Cortland' and 'Empire' (Ju and Bramlage, 1999). These findings show the role played by ROS in inducing superficial scald development.

2.1.6 Anthocyanin content

Anthocyanins are one of the critical antioxidants in physiological disorders. Superficial scald with its causal agents such as conjugated trienes is linked to anthocyanin contents in fruit

(Ingle, 2001). In fact, fruit with high anthocyanin content have been reported having reduced conjugated trienols and low incidence of superficial scald (Barden and Bramlage, 1994). Light intensity is positively correlated with anthocyanin content in the fruit (Dussi et al., 1995; Haselgrove et al., 2000; Oren-Shamir, 2009). Fruit from the same tree greatly vary in anthocyanin content and consequently in scald susceptibility. For instance, outside canopy 'Delicious' apples contained high anthocyanin content and low scalding incidence compared to inside canopy fruit (Ju et al., 1996). Rudell and Mattheis (2009) showed that anthocyanin concentration in 'Granny Smith' apples reduces with increase in scald incidence and severity.

2.1.7 Membrane lipids

Membrane lipids are another intrinsic factor influencing scalding in apples. Scald is expressed as chilling injury prominent in tropical and subtropical crops (Thomai et al., 1998). This is attributed to the time-temperature correlation which governs the expression of scald. Moreover, postharvest techniques used for controlling scald disorder often use the same mode of action as those used to control chilling injury. Membrane lipids play a critical role in reducing chilling stress (Lyons, 1973). The ratio of saturated to unsaturated fats plays an important role in the sensitivity of stored produce to chilling temperatures. Fruit grown in warmer climates have high content of saturated fatty acids in their lipids compared to fruit from cooler climates (Lyons, 1973). Scald often develops when a fruit from warm climate is stored in low temperatures; this might be attributed to the solidification of membrane lipids leading to solid-gel structure (Lafuente et al., 2005), resulting to metabolism imbalances, cell autolysis and subsequently superficial scald.

Additionally, another biochemical disorder that often precedes the expression of symptoms of chilling effects is lipid peroxidation. Lipid peroxidation leads to membrane damage, and consequently browning in form of chilling injury (Lyons, 1973). The resistance of fruit to lipid peroxidation results to low scald incidence. Environmental or storage conditions that retard lipid peroxidation improve lipid accumulation and consequently scald resistance. For instance, Thomai et al. (1998) found high scald resistance in fruit exposed to less than 10 °C for 120-160 h before harvest. Moreover, total lipids, waxes and fatty acids on the peel increased with time of exposure to 10 °C (Figure 2). In fact, increased unsaturated fatty acid content was

recorded on this fruit. Furthermore, during cold storage at 0 °C, unsaturated fatty acids content from fruit that received high chilling units (i.e less than 10 °C for more than 100 h) during pre-harvest was maintained compared to fruit that accumulated low chilling units. Diamontidis et al. (2002) also found increased unsaturated fatty acids content with increasing exposure of fruit to pre-harvest temperatures below 10 °C. The unsaturation of fatty acids in conditions that induce scald resistance has been recorded in previous studies. For instance, Diamontidis et al. (2002) and Thomai et al. (1998) found that exposing fruit at temperatures below 10 °C promotes the accumulation of unsaturated fatty acids compared to fruit not exposed to such conditions. Postharvest studies have also implicated the depletion of membrane lipids during scald development. A recent study by Lu et al. (2014) showed that lipid peroxidation increases with scald incidence and severity in 'Fuji' apples. Actually, early harvested fruit had higher scald incidence and subsequently a higher lipid peroxidation compared to late harvested apples. Shabban-Amin et al. (2011) also reported that electrolyte leakage is more pronounced in scalded 'Granny Smith' apples. Interestingly, scald controlling treatments such as 1-MCP also retard lipid peroxidation (Vilaplana et al., 2006). Moreover, warming 'Fuji' apples at 20 °C for 5 days prestorage at 0 °C for 28 weeks (Lu et al., 2011) increases membrane lipids and consequently reduce scald incidence.

2.1.8 Gene expression

The genetic component coupled with enzyme activity is another predominant intrinsic factor influencing scald resistance and its development in apples. The synthesis and oxidation of α-farnesene which plays a central role in scald development is closely linked to α-farnesene synthase (*AFS1*), an enzyme which converts farnesyl diphosphate to α-farnesene (Rupasinghe et al., 2000; Whitaker, 2004; Sabban-Amin et al., 2011; Busatto et al., 2014). Investigations on α-farnesene biosynthesis pathway have identified ethylene-dependent 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMG2*) and *AFS1*on 'Delicious' and 'Law Rome' apples, respectively, to coincides with marked increase in α-farnesene synthesis (Rupasinghe et al., 2001; Whitaker, 2004). In their investigation on the genetic and enzymatic component of scald development in 'Granny Smith' apples, Lurie et al. (2005) found that an increase in α-farnesene, CTols and subsequent scald incidence is preceded by a marked increase in *AFS1* during storage. Interestingly, *AFS1* transcript was notably lower in 1-MCP treated and non-scalded fruit. In the

same study, even though it was not correlated to scald incidence, the expression of *HMG2* and *HMG3* increased with storage time and marked higher in 1-MCP treated fruit.

Similar genetic variations during cold storage have been reported elsewhere. For instance, Gapper et al. (2006) also found that Pc*AFS1* expression in 'd'Anjou' pears precedes α-farnesene and CTols accumulation. Interestingly, both Pc*AFS1* expression and α-farnesene content are significantly reduced by 1-MCP application (Gapper et al., 2006; Pechous et al., 2005). Other scald controlling treatments such as low oxygen storage have also been linked to expression or inhibition of certain genes. For instance, the expression of ROS scavenging catalase (*MdCAT*) and Mn superoxide dismutase (*MdMnSOD*) was induced in low O_2 and 1-MCP treated 'Granny Smith' apples (Shabban-Amin et al., 2011). Similarly, polyphenol oxidase (*MdPPO*) gene expression was strongly associated with scald development. In fact, its expression was more pronounced in fruit stored in normal air than in fruit treated with 1-MCP or low O_2 before storage.

Recently, the browning in scalded fruit has specifically been correlated to chlorogenic acid accumulation which is activated by *MdPAL*, *MdPPO* and *MdC3H* gene (Busatto et al., 2014). Interestingly, MdPPO which has previously been correlated to scald development (Shabban-Amin et al., 2011) is directly responsible for chlorogenic acid oxidation (Busatto et al., 2014). This new evidence has further revealed that the expression of *MdPAL*, *MdPPO* and *MdC3H*, which coincides with scald development, is reduced by 1-MCP. Notably, the expression of *MdDFR*, *MdANS*, *MdFLS* and *MdLAR* was enhanced by 1-MCP application. These findings show how postharvest treatments 'deactivate' superficial scald responsible genes whilst those that are responsible for scald resistance are 'activated'.

2.2 Extrinsic factors influencing superficial scald development in apples

There are extrinsic factors involved in superficial scald development. Storage temperature, storage time and storage atmosphere are the principal extrinsic factors involved in scald development. The understanding and manipulation of these factors are integral in scald control.

2.2.1 Storage temperature

Storage temperature is the main factor linked to scald. Superficial scald is a low temperature storage disorder (Ingle and D'Souza, 1989; Ingle, 2001). Its occurrence and severity increases with cold storage periods (Brooks et al., 1919; Watkins *et al*., 1995). Scald incidence on apples stored at 20-30 °C have been reported (Brooks et al., 1919). However, scald symptoms are more prevalent at lower temperatures. Like chilling injury, scald symptoms are generally worse at shelf-life. Storage temperatures below 15 °C are reported more conducive for scald development (Ingle, 2001). The time-temperature relationship of scald is similar to chilling injury of subtropical and tropical fruits; in fact, some researchers regard apple scald as chilling injury regardless of apple being a temperate fruit (Watkins et al., 1995). Bramlage and Meir (1990) argued that while temperate fruits are chilling injury resistance, they are not immune hence chilling injury development under rigorous conditions.

2.2.2 Storage atmospheres

Storage atmosphere is another extrinsic factor involved in superficial scald development. Concentration of oxygen and carbon dioxide gases in storage has significant influence on scald development. Postharvest studies have shown that modifying oxygen and carbon dioxide concentration in the storage could reduce scald incidence (Table 5). For instance, cold storing 'Delicious' apples at 0.7% O_2 resulted in lower scald incidence (Lau, 1997a). However, fruit stored in 1.5% O_2 had high scald incidence. The occurrence of CO_2 injury is the major concern of such atmospheres (Johnson et al., 1998). Ventilation and CO_2 removal methods must be optimised to effectively control scald. Moreover, efficacy is affected by cultivar differences (DeEll and Prange, 1998). Moreover, the low market tolerance for scalded fruit requires effective postharvest treatments. Specific cultivar-protocols might have to be developed for effective use of this technology.

3. Superficial scald control

In South Africa, superficial scald is one of the major postharvest physiological disorders reducing the quality of apples. If not controlled, scald result to high postharvest losses and low financial gains. The affected fruit becomes unmarketable (Emongor et al., 1994), it is therefore

pivotal to control this disorder. There are postharvest measures that can be used to reduce scald incidence.

Several postharvest treatments are used to prevent superficial scald in apples. Previous studies have shown that the complexity of pome fruit industries combined with different climatic regions reduces the applicability of a single technique to control scald (Lurie and Watkins, 2012). Varying scales of technological sophistications are cited as the major factor. It is generally advisable to test the given technique before adoption. This is achieved through the help of research institutions in collaboration with fruit industry. Among apple cultivars affected by scald, 'Granny Smith' is probably the worst affected. As a result, 'Granny Smith' is usually used in superficial scald research experiments. Lurie and Watkins (2012) indicated that this might be attributed to the both commercial value and fairly easy identification of scald symptoms on this cultivar. Increased demand for organic food has opened a vacuum in superficial scald research. However, finding non-chemical methods for controlling scald remains a challenge. Below are different postharvest techniques used for controlling scald.

3.1 Chemical treatments

Chemical methods of controlling superficial scald are well reviewed (Lurie and Watkins, 2012). Different antioxidants have been identified for controlling scald in both apples and pears. Antioxidants and ethylene inhibitors have potential to alleviate both abiotic and biotic stresses. Chemical treatments such as DPA, 1-MCP, Ethoxyquin and oils play a significant role in reducing scald incidence. Increased antioxidant pool and phenolic concentration in fruit has been reported after application of DPA and ethoxyquin (Abbasi et al., 2008; Arquiza et al., 2005; Ju and Curry, 2000a). Prevention of α-farnesene oxidation is cited as the major mechanism to scald control. Antioxidant activity influencing α-farnesene oxidation is more critical than α-farnesene concentration on fruit peel (Whitaker et al., 2000).

3.1.1 Diphenylamine (DPA)

DPA was probably the most popular and widely used antioxidant controlling scald in pome industry. There is ample literature evidence showing the efficacy and effectiveness of DPA in retarding superficial scald (Table 6). For example, Moggia et al. (2010) reported reduced scald

incidence in 'Granny Smith' apples stored at 0 ^{0}C for six months. In investigating the influence of delayed DPA application on scald development, Jung and Watkins (2008) and Rudell et al. (2009) reported that DPA efficacy to retard scald is not compromised by delayed application. The reduction of CTols and MHO from α-farnesene oxidation is the major mechanism used by DPA to alleviate scald (Isidoro and Almeida, 2006; Whitaker, 2000). Moreover, the reduction of ethylene production during storage after DPA application has also been reported (Jung and Watkins, 2008). Additionally, Lurie et al. (1990) reported different metabolic responses after DPA application. Reduced activities of polyphenol-oxidase, peroxidase and lipoxygenase coupled with decreased ethylene production were recorded.

Although DPA is highly effective in controlling superficial scald, environmental concerns have been raised regarding its use. As a result, the European Union (EU) Commission reduced the MRL from 5 parts per million (ppm) to 0.1ppm (APAL, 2013; Crouch and Taylor, 2013). The current and future research should be focused on finding effective postharvest treatments that could offer complete scald control like DPA.

3.1.2 1-Methlycyclopropene (1-MCP)

1-MCP is also an effective superficial scald inhibitor (Fan et al., 1999; Isidoro and Almeida, 2006; Watkins et al., 2000). Mechanisms of action used by DPA and 1-MCP to reduce scald incidence are distinctly different (Jung and Watkins, 2008). In contract to inhibition of α-farnesene oxidation and CTols accumulation by DPA, 1-MCP inhibits α-farnesene accumulation and indirectly retards CTols and MHO (Isidoro and Almeida, 2006). Moreover, the highly involved ethylene gas in superficial scald biochemistry is inhibited by 1-MCP. Previous studies have shown the efficacy and effectiveness of 1-MCP to control scald (Table 6). For instance, 1-MCP application completely controlled scald in 'Granny Smith' apples stored at 0 ^{0}C for 16 weeks; however, control fruit developed scald just after 8 weeks (Shaham et al., 2003). Moreover, fruit applied with 1-MCP had high lipid soluble antioxidant activity and consequently no scald incidence.

Dauny and Joyce (2002) working on 'Queen Cox' and 'Bramley' apple found a reduced ethylene production and subsequently low scald incidence on 1-MCP treated fruit. The effect of

1-MCP to control scald is dependent on many factors including cultivar and maturity. Fruit harvested at different maturities cannot be stored together as this compromises the effect of the treatment. 1-MCP completely controls scald in 'Granny Smith' (Zanella, 2003; Lurie et al., 2005), however, storage type and duration play may affect its efficacy for many other cultivars (Fan et al., 1999; Watkins et al., 2000; Bai et al., 2006). Reduced scald incidence, retained fruit firmness and acceptable ripening have been realised in such treatments. However, this strategy might have to be replicated in other production areas as climate plays a significant role in effectiveness of postharvest treatments. For instance, Ju and Watkins (2008) reported delayed 1-MCP application to be less effective in controlling scald. Combining delayed 1-MCP application with CA or initial low oxygen stress (ILOS) may perform best in some fruits especially during longer term storage at low temperature. Innovative strategies providing a balanced scald control and acceptable fruit quality post 1-MCP treatments should be developed.

3.1.3 Plant oils

Oils can also inhibit superficial scald incidence in pome fruits. The usage of fruit paper wrappers impregnated with oils is an ancient method used since early 20th century (Brooks et al., 1919; Hall et al., 1953). Fruit wrappers with 15% mineral oil have previously been used at commercial scale for decades in controlling superficial scald (Brooks et al., 1919). However, increased labour costs have made this method uneconomic and unpractical. Recent research has focused on the effects of plant-based oils also known as essential oils on controlling scald (Scott et al., 1995; Ju and Curry, 2000b). Sunflower; castor, canola, palm or peanut oils reduced scald in 'Granny Smith' apples stored at 0 °C for 19 weeks (Scott et al., 1995). However, fruit treated with castor or palm oil had reduced quality due to greasiness. Contrary, oils extracted from canola, sunflower and peanut exhibited best quality fruit poststorage. Essential oils from soybean, corn, olive, linseed and cottonseed have also been proved to be highly effective. Ju et al. (2000) found reduced scald incidence (<4%) in 'Delicious' apples treated with these oils prestorage. However, α-tocopherol extracted from corn oil was proved to be very effective compared to other oils. In fact, scald in 'Granny Smith' apples was effectively controlled by this oil compared to DPA. Essential oils may reduce membrane permeability and respiration rate, and further promote the migration of α-farnesene from fruit to oil wrappers (Lurie and Watkins, 2012). For instance, reduced ethylene and α-farnesene production in apples treated with

vegetable oil prestorage has been reported (Scott et al., 1995; Ju and Curry, 2000b). However, there is limited investigation into the effects of essential oils on scald control. With DPA posing environmental concerns, more research has to focus on the optimisation of essential oils. Moreover, best methods for applying essential oils should be developed.

Reduced scald incidence and unacceptable fruit quality has been reported after essential oil application on apples. For example, dipping 'Granny Smith' and 'Jonathan' apples in a mixture of castor oil and shellac retarded scald; however, fruit had skin blemishes and produced off-flavours (Hall et al., 1953). In contrast, fruit wrapped with the same oils in the same study had high quality. Scott et al. (1995) also reported a reduced scald incidence in 'Granny Smith' apples due to the application of palm soil; however, fruit quality was compromised as the fruit was oily and greasy with more visible lenticels. Advances in postharvest engineering could be instrumental in designing new equipment and developing protocols for optimum application of essential oil to minimise the negative impacts on fruit quality. Scott et al. (1995) proposed that absorbent rollers could be used to remove excess oil on fruit surface. Volatile evolution and palatability after essential oil application should also be investigated.

3.2 Non-chemical treatments

Chemical treatments of fresh produce are becoming less acceptable to consumers; alternative methods for controlling physiological disorders are urgently required. Although previous research has demonstrated the effectiveness of synthetic antioxidants such as ethoxyquine and DPA in controlling scald. However, the negative effects imposed by chemical treatments on human health and environment have restricted their use (Lau, 1997a; Kim-Kang et al., 1998). Additionally, chemical treatments are forbidden in organic markets. Current superficial scald research is focused on finding non-chemical treatments for controlling scald. Potential methods have been developed by postharvest researchers and engineers. These methods include ventilation, heat treatment (HT), intermittent warming (IW), controlled atmosphere (CA), initial low oxygen storage (ILOS) and more recently, dynamic controlled atmosphere (DCA).

3.2.1 Ventilation

Airflow patterns during cold chain control influence storage temperature and relative humidity and subsequently prolong shelf-life of stored produce (Opara and Zou, 2006). Moreover, optimal designs and efficacy of the cooling process are paramount in reducing postharvest losses of perishable products (Pathare et al., 2012). In fact, excessive or insufficient venting compromises the quality of produce (Opara, 2011). Previous studies have shown a close relationship between ventilation and scald incidence in apples (Brooks, 1923; Huelin and Coggiola, 1970; Sfakiotakis et al., 1993; Watkins and Thompson, 1992). Packaging systems and material that restrict air flow create conducive environment for scald development. For example, the use of commercial polybags or microperforated polybags in 'Cox' apples stored at 1 and 3 ^{o}C reduced ventilation and increased scald development (Watkins and Thompson 1992). Recent studies have shown that storing 'Granny Smith' apples in flow-through CA storage inhibits scald for up 8 months (Wang and Dilley, 2000a). However, the effect of ventilation on bioactive compounds remains complex. Alpha-farnesene evaporation and reduced scald incidence is highly correlated with high air flow rate around the fruit (Anet 1972; Huelin and Coggiola 1970). More, reduced α-farnesene oxidation to either MHO or CTols was also reported. However, Wang and Dilley (2000b) reported that reduced scald incidence due to ventilation cannot solely be explained by α-farnesene, the hindered accumulation and loss of scald-related volatiles could play a critical role. Further research on the effect of ventilation on evolution of volatile compounds and phytochemicals is warranted. Moreover, incorporating 'optimised' ventilation system to existing technologies could be a major breakthrough in scald research.

3.2.2 Heat treatment

The use of heat treatments to increase storage potential of horticultural produce is an ancient practise. Exposing fruit to high temperatures during storage induces resistance to chilling stresses such as chilling injury and superficial scald. Jemric et al. (2006) reported that hot water treatments at 48 ^{0}C are very effective in retarding superficial scald in 'Granny Smith' apples. Additionally, water dips of 10 min at 40 ^{0}C or 50 ^{0}C for 5 min have also been demonstrated to retard scald incidence (Lurie and Watkins, 2012). Moreover, hot air treatment (38 ^{0}C) of 4 days reduced scald development in 'Granny Smith' apples (Klein and Lurie, 1992; Lurie et al., 1990).

Although heat treatments are effective in controlling scald, their mechanism of action is unclear and not yet understood. Heat treatments may inhibit α-farnesene accumulation and oxidation (Fallik et al., 1997; Lurie et al., 1990; Lurie et al., 1991; Shaham et al., 2003) and may also delay ethylene production (Lurie and Klein 1990). Additionally, the accumulation of heat shock proteins (Lafuente et al., 1991) and hydrophilic antioxidants (Shaham et al., 2003) have been reported. Conversely, Lurie et al. (2005) did not find heat treatment consistent with longstanding hypothesis of α-farnesene and its oxidation products. Moreover, Shaham et al. (2003) detected inconsistent trends in evolution of both lipophilic antioxidant and enzyme activity. The inconsistence of results in heat treatments warrants further research as no conclusion can currently be made. Antioxidant accumulation, enhanced gene expression (heat shock proteins) and enzymes might be important factors involved in mechanism of action used by heat treatment. Although heat treatments have potential to control scald, its adoption in pome industry remains a challenge. This is probably due to uneconomical energy requirements of this method and inconsistency in efficacy, sanitation and current water free systems on pears due to high decay development. Development of energy efficient systems might probably lead to its commercialization. Moreover, further studies on the effect of heat treatments on scald biochemistry will improve the understanding of superficial scald and subsequently optimise currently used technologies.

3.2.3 Intermittent warming

Intermittent warming (IW) is another non-chemical technique that retards physiological disorders in horticultural produce. Superficial scald is reportedly inhibited by IW treatments. Alwan and Watkins (1999) investigated the potential of IW to control superficial scald 'Cortland', 'Delicious' and 'Law Rome' apples. The study found that weekly IW treatments at 20 °C controlled scald and prolonged shelf-life. Similarly, Watkins et al. (1995) observed a reduced scald incidence in 'Granny Smith' apples warmed at 15 ^{0}C or 20 ^{0}C for 5 consecutive days and later stored at 0 ^{0}C for 25 weeks. Additionally, Lu et al. (2011) found similar effects on 'Fuji' apples intermittently warmed for a single 5 days at 20 ^{0}C. All these findings highlight the potential inherent in the use of IW to maintain fruit quality and control scald. However, the influence of cultivar, environmental conditions and seasonal variations plays a critical role in IW

efficacy (Watkins et al., 2000a). This might be attributed to the effect of climatic conditions on skin permeability, wax composition and volatile composition (Watkins et al. 2000a).

Based on these findings, it is clear that IW is effective in controlling scald, however, mechanism of action remains unclear. Increased antioxidant pool in the fruit peel is implicated as a possible IW mechanism (Lu et al., 2011; Watkins et al., 1995). Alwan and Watkins (1999) investigated the effect of IW on α-farnesene and CTols evolution. The study observed CTol concentrations to be inconsistent with scald development. Actually, CTols and scald were negatively correlated. Intermittent warming could also affect scald by inducing membrane resistance to the damaging properties of ROS, MHO and CTols. Further research is necessary to understand the IW mechanism of action. Impaired fruit quality is a major setback for some cultivars. For instance, Johnston et al. (2005) reported softening in 'Royal Gala' and 'Cox' apples temporarily transferred from 0 °C to ambient temperatures. Weekly warming 'Cortland' apples at 20 ^{0}C increased IEC and subsequently firmness loss after 22 weeks cold storage (Alwan and Watkins, 1999). This method can be improved to make it attractive and sophisticated whilst ensuring good quality fruit poststorage. Real-time sensing technology that could give signals when IW is about to induce 'stress' and automatically reduce temperatures should be exploited. Internal ethylene concentration, due to its inherent effect on fruit firmness, could play a major role in modelling such technology.

3.2.4 Controlled atmosphere (CA)

The manipulation of storage atmospheres is a common method used to control scald incidence in both pears and apples. Decreasing available oxygen (O_2) levels and increasing carbon dioxide (CO_2) of the stored produce reduces scald incidence (Lurie and Watkins, 2012; Wright et al., 2015). There are critical factors that should be taken into consideration for effective CA technology. Previous studies have shown that cultivar, fruit maturity, storage duration and ventilation determine the success of CA technology (DeEll and Prange, 1998; Wang and Dilley, 1999; Whitaker, 2000). For instance, Lau (1990) and Lau (1997a) reported scald control (less than 10%) in 'Delicious', 'Starking' and 'Harrold Red' apples stored at CA of 0.7% O_2 and 1% CO_2, however, growing areas and seasonal variation had extrinsic effect on CA efficacy. The efficacy of CA was further influenced by cultivar and fruit maturity. While

'Starking' and 'Harrold' apples picked over a wide range of maturity had less than 10% scald incidence following CA storage, Starkrimson' had 45% scald incidence. DeEll and Prange (1998) also reported that storage atmosphere of 1.5% or 2.5% O_2 cannot control scald in 'Cortland' apples. These results indicate that gas composition in CA storage could be environmental, maturity and cultivar specific. On the impact of ventilation, storing apples in low oxygen and flow-through CA system is more effective in controlling scald compared to static CA system (Wang and Dilley, 1999; Whitaker, 2000).

Controlled atmosphere storage is associated with low scald incidence, suppression of ethylene production, α-farnesene and its oxidation products. Storing 'Granny Smith' apples in CA of 1% O_2 + 2% CO_2 at 0 ^{0}C for 205 days reduced scald to 20% compared with 90% in control fruit (Piretti et al., 1994). The reduction of scald coincided with stable polyphenols contents, contrary, polyphenol content declined in control fruit. Controlled atmospheres play a critical role in lowering ethylene production, thereby reducing the accumulation of toxic volatiles such as α-farnesene and its oxidation products, CTols and MHO (Piretti et al., 1994; Whitaker, 2000).

The major setback of CA technology is the risk of off-flavours due to low oxygen concentrations. Low oxygen concentrations might trigger anaerobic respiration resulting to suppression of aroma production and subsequently off-flavours (Mattheis et al., 1998). The static controlled atmospheres cannot detect the low oxygen concentrations just above anaerobic threshold (DeLong et al., 2007; Zanella, 2003). Improved CA technology may probably present more advantages compared with the current CA technology. Moreover, combining CA technology with other treatments such as 1-MCP or initial low oxygen stress (ILOS) maybe beneficial. For example, Wang and Dilley (2000) reported a reduced scald incidence in fruit pre-treated with ILOS prior CA storage. Similarly, CA storage of 1.5 kPa O_2/3 kPa CO_2 or 1 kPa O_2/1 kPa CO_2 proceeded by 10 to 14 days ILOS treatment (0.4 kPa O_2) controlled scald in 'Granny Smith' apples (van der Merwe et al., 2003; Zanella, 2003). However, combining ILOS and CA could be very expensive for producers (Wang and Dilley, 2000b; Zanella, 2003).

Similarly, chemical treatments are combined with CA for better efficacy in reducing scald. A combination of CA and ethanol vapour reduced scald incidence in 'Granny Smith'

apples stored at 1 ºC for 5 months (Chervin et al., 2001). Similarly, the treatment of 'Granny Smith' apples with DPA (1800 mg l^{-1}) before CA storage at 1 kPa O_2 + 1 kPa CO_2 completely controlled scald (Zanella, 2003). However, DPA is not permitted by certain importing countries; innovation strategies to enhance CA performance are thus needed. Watkins et al. (2000) demonstrated that treating 'McIntosh' and 'Law Rome' with 1-MCP before CA storage controls superficial scald. However, in the same study, all 1-MCP concentrations (0.5 to 2 µl l^{-1}) prevented ripening in 'Delicious' and 'Empire' apples. Combining 1-MCP and CA was more effective in retaining the quality and firmness of 'Pink Lady' apples stored at 2 ^{0}C for 6 months (Cocci et al., 2014). DeEll and Ehsani-Moghaddam (2013) also reported the similar findings in 'McIntosh' and 'Spartan' apples stored at 2 ^{0}C for 6 months. However, the efficacy of the treatment was only effective for 6 months as the quality reduced after extending storage to 9 months. Similar to ILOS, the 1-MCP costs may escalate the expense of this technology should it be effective. The cost ratio of 1-MCP to DPA is roughly 10:1. If all apples would be treated with 1-MCP, there would be an additional cost of R30 million on the total fruit stored (J.A van der Merwe, personal communication). Reducing 1-MCP concentration and optimizing CA gas composition could be highly beneficial.

3.2.5 Initial low oxygen stress (ILOS)

The potential of initial low oxygen stress (ILOS) to inhibit superficial scald has been investigated (Wright et al., 2015). The discovery of ILOS to control scald dates back to early 1980s (Little et al., 1982). Scald control after ILOS treatment has been reported in both apples and pears. Generally, ILOS is conducted either at 0 °C or 20 °C prior regular cold storage or CA storage. A couple of studies have investigated the effect of ILOS done at 20 °C. For instance, Ghahramani and Scott (1998) and Pesis et al. (2010 and 2014) investigated the effect of short anaerobiosis period prior cold storage on 'Granny Smith' apples. The study reported a retarded scald incidence after storage. Similarly, a complete scald control in 'Granny Smith' apples treated with 0.5% O_2 for 10 days before 0 °C storage of 24 weeks has been reported (Sabban-Amin et al., 2011). The major setback of ILOS conducted in room temperature or 20 °C is high production of anaerobic metabolites such as acetaldehyde and ethanol (Ghahramani and Scott, 1998; Pesis et al., 2010).

Other studies have done ILOS treatment at 0 °C prior long term cold storage. For instance, Wang and Dilley (2000b) reported a complete scald control in 'Granny Smith', 'Law Rome' and 'Red Delicious' apples pretreated with 0.25% or 0.5% O_2 for 2 weeks and subsequently stored in CA (1.5% O_2, 3% CO_2) for 8 months. In contrast, Lau (1997b) reported that ILOS offers no scald control benefits to 'Starkrimson Delicious' apples in 0.7 kPa O_2 storage. It is clear that the efficacy of ILOS could be influenced by cultivar. Unlike ILOS treatments conducted at 20 °C, other quality attributes such as reduced water-core breakdown have been reported in ILOS conducted at 0 °C (Lau, 1997b). Fruit maturity has also been shown to influence the ILOS efficacy to control superficial scald and other physiological disorders (Lau, 1997b). In 'Starkrimson Delicious' apples, ILOS is more effective in optimal harvested fruit (Lau, 1997b). Research efforts should be concerted on developing cultivar and maturity specific ILOS protocols. This will ensure a complete scald control during cold storage.

Previous studies have made attempts of understanding how ILOS inhibit scald development in apples. One possibility could be conversion of the dangerous molecule MHO, which is a ketone, to a non-dangerous molecule, alcohol 6-methyl-5-hepten-2-ol, which is an alcohol (Pesis et al., 2010). The inhibition of ethylene accumulation and consequently α-farnesene and MHO production has also been reported in scald free fruit stored in ILOS (Ghahramani and Scott, 1998; Pesis et al., 2014; Sabban-Amin et al., 2011). In fact, Sabban-Amin et al. (2011) reported a 70-fold higher α-farnesene content in control than fruit pretreated with ILOS before storage. Ethanol accumulation could possible be involved in the mechanism of action for ILOS. Ghahramani and Scott (2000) and Pesis (2005) reported a strong correlation ethanol accumulation and reduced ethylene and α-farnesene in ILOS treated fruit.

3.2.6 Dynamic controlled atmosphere (DCA)

Recent research findings have established the potential and efficacy of DCA technology to control scald and prolong storage life of apples and pears. Unlike the static atmosphere imposed by CA, DCA is based on changing the storage atmosphere (Wright et al., 2012; Yearsley et al., 2003; Thewes et al., 2015). Gas composition of CA storage varies depending on the product or the container used; atmospheres normally range from 2 to 5% O_2 and 3 to 10% CO_2 (Yearsley et al., 2003). Contrary, in DCA the O_2 levels change depending on the response of

fruit which varies during storage, to achieve this, a non-destructive sensor measuring chlorophyll fluorescence or respiration quotient linked to the O_2 adjuster monitors the physiological response of the fruit (Yearsley et al., 2003; Weber et al., 2015; Wright et al., 2015). The DCA technology is based on the lower oxygen limit (LOL) at which cell metabolism of the fruit shifts from aerobic to anaerobic (Wright et al., 2012). The LOL is identified by slowly reducing the O_2 level until the detection of O_2 stress (Burdon et al., 2008; Wright et al., 2012). To alleviate the risk of irreversible damage from O_2 stress, the sensor output trigger the CA control system to automatically increase O_2 level.

Recent research has demonstrated the potential of low oxygen levels to control scald and retain fruit quality (Thewes et al, 2015). Prange et al. (2011) and Wright et al. (2015) indicated that DCA can prolong storage life and inhibit scald in both apples and pears. Zanella et al. (2005) on their research entitled "Fruit fluorescence response to low oxygen stress: modern storage technologies compared to 1-MCP treatment of apple" reported a complete scald control in DCA stored 'Granny Smith' apples after a 6-month cold storage and 14 days shelf-life. Contrary, 35% scald incidence was recorded in 'Cortland' apples stored at DCA whilst static CA had 85% scald incidence (DeLong et al., 2007). Recent research efforts on 'Greenstar' (Tran et al., 2015), 'Galaxy' (Thewes et al., 2015) and 'Royal Gala' apples (Thewes et al.; Weber et al., 2015) have also shown that DCA is highly effective in ensuring maximum quality retention during storage. Although recent findings about the potential DCA to inhibit scald are highly significant in fruit industry, the mechanism of action is yet to be investigated. In recent attempts of understanding the mechanism of action, DCA has been linked to reduced oxidative metabolism resulting to decreased 1-Aminocyclopropane-1-Carboxylate (ACC) oxidase activity and subsequently ethylene production (Weber et al., 2015). More research should be conducted for all the scald susceptible cultivars. This will be useful in developing cultivar specific protocols for DCA storage.

The influence of DCA on fruit quality is not fully known. Zanella et al. (2005) detected no sign of low oxygen disorder neither off-flavour in DCA stored fruit. In the same study, 1-MCP delayed degradation of organic acids and subsequently resulted to higher TA after shelf-life, however, DCA stored fruit had superior organoleptic properties. 'Cortland' apples stored at DCA had higher firmness retention; moreover, both total TSS and TA remained high (DeLong et

al., 2007). However, internal browning and breakdown has been reported in 'Abbè Fetél' pears stored in DCA (Vanoli et al., 2010). Recently, Tran et al. (2015) reported no significant differences in sensory quality of 'Greenstar' apples stored in DCA or static CA. In contrast, Thewes et al. (2015) reported that flesh firmness and superior quality in 'Gala' and 'Galaxy' apples stored in DCA compared to those stored either in CA or ULO. Further research is required to study the influence of DCA on fruit quality and organoleptic properties.

4. Emerging technologies and future prospects

Developing non-chemical treatments for controlling superficial scald in apples and pears has been the epitome of postharvest research for decades. Prestorage treatments such as heat treatments (i.e hot water dips or hot air treatment), intermittent warming and controlled atmospheres have been developed. However, their inability to completely control scald (compared to DPA) and high cost remains a major challenge. The development of acceptable postharvest practices and treatments that provide DPA-like scald control for the apple industry is the necessity in scald research (DeLong et al., 2007). A lofty shift from postharvest treatments to planting resistant material must occur. A deeper and broader understanding of ethylene synthesis and its evolution will play a major role in achieving this objective. More research is required to elucidate the real impact, if there is, of CTs on scald. Another area of interest yet to be exploited is discovering whether there is correlation between fruit maturity and CTs accumulation.

4.1 Advancing DCA research

Although DCA technology has been commercially used since 2004 (Prange et al, 2011; Wright et al., 2015), published research information about DCA technology remains limited. Very limited research has focused at the feasibility of using DCA in an apple industry, particularly for storing 'Granny Smith' apples. Moreover, apple industries, globally and within countries, vary in orchard management practices and levels of technological sophistication. In the context of Italy, a leading DCA technology user, the markets (mainly in Europe) are close and the fruit is transported from DCA storage directly to the markets, however, some markets are distant and necessitates shipment period of 6 weeks (fruit is stored at 0 ^{o}C, regular atmospheres). Unexpected demand of fruit often necessitates the opening of storage facilities before the projected time. Studies on DPA treatments revealed that intermittent breaks do not affect the

effectiveness of treatments. However, it remains unclear if DCA effectiveness is not compromised by intermittent breaks. Moreover, the mechanism of action for DCA is not yet understood. All these questions mandate more DCA research in apples. Biochemical experiments are thus required to study the influence of DCA on evolution of scald related metabolites such as ethylene, α-farnesene, CTols and MHO. The possible involvement of ROS, lipid peroxidation and enhanced antioxidants pool in DCA stored fruit should not be ignored.

4.2 Modelling superficial scald

Previous research has identified both extrinsic and intrinsic factors that are involved in scald development. Ethylene, MHO, CTols, phytochemicals, storage time and storage atmospheres are amongst the principal factors determining scald susceptibility. Future research should focus on the utilization of these factors in applying mathematic modelling to predict scald development and incidence. Recent postharvest technology and engineering research has proved that evaluating the quality of the final product through mathematical simulation is achievable. Actually, prediction models have become more accurate in predicting the outcome of complex issues such genotype-environment interactions (Vázquez-Cruz et al., 2010). Such tool enables identification of critical points during postharvest handling and subsequently improve decision making. A prediction model for superficial scald that is mechanistic enough to provide real-time description of physiological changes and variations in quality parameters should be prioritized.

Table 7 presents a summary of articles on superficial scald, highlighting the cultivar, experimental parameters and model developed. Most superficial scald models have been oriented towards using accumulative night temperature units below 10 °C. However, recent developments in scald prediction models have focused on postharvest physiological parameters. The regression equations based on night hours below 10 °C, days after full bloom and optimal density (OD) 200nm absorption of hexane extracts (Bramlage et al., 1993; Barden and Bramlage, 1994; Thomai et al., 1998; Lourens and Malherbe, 1997), ratio of α-farnesene to CTs (Zhang and Shu, 2003), and rate of conjugated trienes accumulation (Giné Bordonaba et al., 2013) have been reported to predict scald incidence. Using climatic data, Lourens and Malherbe (1997) developed scald prediction models; however, the models were inconsistent in predicting scald as climatic differences had a significant effect. They proposed that developing a model for a specific climate

would be appropriate. However, focus should be given to developing a standard prediction model that can provide reliable results regardless of preharvest conditions of fruit.

Zhang and Shu (2003) investigated the feasibility of developing a scald prediction model on 'Starking' and 'Ralls' apples harvested at different maturities. The study successfully developed a scald prediction model based on the ratio of α-farnesene to CTols as a reliable predictor of scald development in fruit. However, high CTols, regardless of low scald incidence in late harvested fruit, reduced the precision of this model. Recently, a scald model based on the rate of conjugated trienes accumulation (Giné Bordonaba et al., 2013) has been developed and validated. This model was described as sensitive and having good prediction power; however, it is only based on 'Granny Smith' apples and further studies are required to examine the suitability of this model for other apple and pear cultivars. Moreover, different climatic and storage conditions or technologies can influence the behaviour of a prediction model. In addition, even though MHO is one of the products of α-farnesene oxidation that is strongly associated with scald development (Wang and Dilley, 2000a), no scald prediction model has included it (Table 7). It is therefore clear that further research is warranted. All these findings highlight the potential of developing a standard, reliable and real-time scald prediction model. Ingle (2001) indicated that a robust prediction model should include almost all the factors involved in scald development. Concerted research effort should therefore be made to develop simple but robust models that possibly combine α-farnesene, CTols, MHO and phytochemicals for predicting superficial scald incidence.

4.3 Non-destructive measurements

The next step post prediction model development should be investigating the feasibility of using non-destructive methods such as near-infrared radiation (NIR) technology for quantifying scald-related volatiles and phytochemicals. NIR reflectance spectroscopy has recently been successfully used as a predictive tool for respiration rate and respiratory minimum in apples (Touchant et al., 2000). Nicolaï et al. (2008) correctly predicted soluble solids content and firmness of 'Conference' pear using NIR reflectance spectroscopy. Recently, optical coherence tomography (OCT) has proved to be feasible technique for real-time and non-destructive acquisition of images showing histological and microstructural rind features of

'Nules Clementine' mandarin (Magwaza et al., 2013). This findings show the possibility of developing prediction models that could be based on non-destructive techniques for predicting superficial scald in apples.

The ability of "index of absorbance difference" (IAD), as measured with a DA-Meter, a portable device based on NIR, to check fruit maturity and predict scald development (Farneti et al., 2015) has shown that non-destructive techniques for quantifying scald-associated metabolites should be the epitome of scald research in the next decade. This rapid and non-destructive method showed scald incidence to be closely correlated to ripening stage, generally, immature fruit showed higher scald development. Farneti et al. (2015) further indicated that IAD could be integrated into pack-line to optimise postharvest management. Developing prediction models and non-destructive techniques for immediate and real-time physiological and microstructural changes in apple fruit is important for scald research, and in developing postharvest handling protocols. The utilization of non-destructive techniques to predict scald development is very critical as the pressure is mounting on reducing chemical usage, moreover, the cost of research could greatly reduce.

5. Conclusions

In spite of the progress made in understanding superficial scald biochemistry and physiology, the mechanisms of action of certain treatments for controlling the disorder remain complex and not well understood. DCA is a recent technology that shows potential in retarding scald incidence. However, its mechanism of action is yet to be understood. In fact, a series of questions are yet to be answered. The influence of factors such as cultivar, fruit maturity, storage conditions and intermittent breaks on DCA efficacy has not yet been investigated. More research is therefore warranted.

This review has shown that controlling scald with only non-chemical methods is not farfetched. It is clear that historically more focus has been given into understanding the relationship of α-farnesene and its oxidation products to superficial scald, the role of antioxidants and reactive oxygen species such as hydrogen peroxide and superoxide has received little attention. A holistic approach should be taken in studying and understanding factors influencing scald. Such information will be vital in developing simple, reliable and robust scald prediction

models. Although some prediction models have been developed, there are questions yet to be answered. In addition, the assertion that each pome industry should develop its prediction model due to differences in climate (Lourens and Malherbe, 1997) should be considered. No single cold storage method or treatment is applicable across the range of pome fruit industries around the globe given that each industry has varying orchard practice methods and different levels of technological sophistication. However, efforts should be concerted on finding a suitable prediction model that might be applicable to every industry. The feasibility of measuring scald-related volatiles non-destructively should be exploited. This will reduce the time, budget and environmental costs involved in answering some physiological questions. Moreover, the use of chemical treatments in postharvest systems could be greatly reduced.

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Table 1

Selected research review papers on superficial scald.

Reference	Scope of the review	Product of focus
Smock (1961)	Advances in postharvest methods	Apple
Meigh (1970)	Biochemistry of superficial scald	Apple
Wilkinson and Fidler (1973)	Understanding the biology of superficial scald	Apple and pear
Barlow (1988)	Interpreting the physiology and biology	Apple and pear
Ingle and D'Souza (1989)	Advances in understanding physiology and inhibition of superficial scald of apples	Apple
Emongor et al. (1994)	Climatic and preharvest factors influencing scald	Apple
Wang and Dilley (1999)	Preharvest and postharvest factors influencing scald and control	Apples
Ingle (2001)	Current understanding of superficial scald and future prospects	Apple and pears
Lurie and Watkins (2012)	Etiological understanding of superficial scald and its control	Apple and pears

Table 2

Intrinsic and extrinsic factors influencing superficial scald development in apples.

Intrinsic factors	**Extrinsic factors**
Cultivar	Storage temperature
Fruit maturity	Storage time
Ethylene content	Storage atmosphere
Volatile composition	
*α-farnesene	
*6-methyl-5-hepten-2-one (MHO)	
*Conjugated trienols	
Phytochemicals and antioxidant content	
Membrane lipids	

Table 3

Selected examples of apple cultivars that differ in superficial scald disorder.

Cultivar	Scald Susceptibility	Country	Reference
'Granny Smith'	Susceptible	Italy, New Zealand, Greece, USA, South Africa, Australia, Croatia, Belgium, Israel	Golding et al. (2001); Jemric et al. (2006); Piretti et al. (1994); Rudell et al. (2009); Sabban-Amin et al. (2011); Thomai et al. (1998); van der Merwe and Combrink (2001); Watkins et al. (1995); Watkins et al. (2000); Farneti et al. (2015)
'Law Rome'	Susceptible	USA	Alwan and Watkins (1999); Pechous and Whitaker (2004); Whitaker (2004); Pechous et al. (2005)
'McIntosh'	Susceptible	Canada	Ahn et al. (2007)
'Idared'	Susceptible	Canada	Ahn *et al*. (2007)
'Cortland'	Susceptible	USA, Canada	Ahn et al. (2007); DeEll et al. (2002); Watkins et al. (2000)
'Bramley'	Susceptible	UK	Dauny and Joyce (2002)

'Red Delicious'	Susceptible	USA	Whitaker (2004)
'Empire'	Resistant	USA, Canada	Ingle (2001); DeEll et al. (2002); Ahn et al. (2007)
'Royal Gala'	Resistant	New Zealand, USA, Canada	Ahn et al. (2007); Beuning et al. (2010); Mattheis et al. (1998)
'Golden Delicious'	Resistant	Italy, USA	Ingle (2001); Zanella et al. (2008)
'Crofton'	Resistant	Australia	Golding et al. (2001)

Table 4

Analytical methods used for quantifying α-farnesene, conjugated trienols (CTols) and 6-methyl-5-hepten-2-one (MHO).

Volatile compound	Method	Reference
α-farnesene	Spectrophotometer	Anet, 1972; Chen et al., 1990; Du and Bramlage, 1994; Gong and Tian, 1998; Isidoro and Almeida, 2006; Jung and Watkins, 2008; Lu et al., 2011; Moggia et al., 2010; Shaham et al., 2003; Tsantili et al., 2007
	HPLC	Gapper et al., 2006; Whitaker, 2000a; Watkins et al., 2000b;
	GC-MS	Golding et al., 2001; Ju and Curry, 2002; Mir et al., 1999;Rudell et al., 2005; Wang and Dilley, 2000a; Pesis et al., 2009, 2010; Sabban-Amin et al., 2011; Busatto et al., 2015; Farneti et al., 2015
Conjugated Trienols	Spectrophotometer	Alwan and Watkins, 1999; Chen et al., 1990; Isidoro and Almeida, 2006; Jemric et al., 2006; Lu et al., 2011; Moggia et al., 2010; Shahan et al., 2003; Tsantili et al., 2007
	HPLC	Gapper et al., 2006; Watkins et al., 2000a; Whitaker, 2000b
	GC-MS	Rudell et al., 2005
MHO	GC-MS	Fan et al., 1999; Ju and Curry, 2002; Mir et al., 1999; Wang and Dilley, 2000a; Pesis et al., 2009, 2010; Sabban-Amin et al., 2011; Busatto et al., 2015; Farneti et al., 2015

Table 5

Gas composition of storage atmosphere for apple storage.

Cultivar	**O_2**	**CO_2**	**Temp.**	**Reference**
'Granny Smith'	1%	2%	0^0C	Piretti et al. (1994)
'Starkrimson Delicious'	0.7%	1%	0^0C	Lau et al. (1998)
'Cortland'	1.5 kPa	1.5 kPa	0^0C	Dell and Prange (1998)
'Bramley'	1 kPa	5 kPa	4^0C	Colgan et al. (1999)
'Law Rome'	1.5%	3%	0.5^0C	Wang and Dilley (2000)
'Granny Smith'	2 kPa	1 kPa	1^0C	Chervin et al. (2001)
'Granny Smith'	1.5 kPa	1.3 kPa	1.3^0C	Zanella (2003)
'Granny Smith'	2%	3%	0^0C	Erkan et al. (2004)
Granny Smith	0.7 kPa	0.5 kPa	0.5^0C	Giné Bordonaba et al. (2013)
'Spur Red Delicious'	0.3 kPa	1 kPa	0.5^0C	Lumpkin et al. (2014)

Table 6

Selected papers on effect of DPA and 1-MCP treatment on scald alleviation.

Chemical	Cultivar	Scope of the research	References
DPA	'Empire'	DPA treatment alters a-farnesene metabolism in peel of 'Empire' apples stored in air or 1.5% O_2 atmosphere	Whitaker (2000)
	'Granny Smith'	Relationship between production of ethylene and α-farnesene in apples, and how it is influenced by the timing of diphenylamine	Golding et al. (2001)
	'Granny Smith'	Control of apple superficial scald and ripening-/a comparison between 1-methylcyclopropene and diphenylamine postharvest treatments, initial low oxygen stress and ultra-low oxygen storage	Zanella (2003)
	'Granny Smith'	Relationship of superficial scald development and α-farnesene oxidation to reactions of diphenylamine and diphenylamine derivatives in cv. Granny Smith apple peel	Rudell et al. (2005)
	'Empire'	Influence of 1-methylcyclopropene (1-MCP), diphenylamine (DPA), and CO_2 concentration during storage on 'Empire' apple quality	DeEll et al. (2002)
	'Cortland'	Superficial scald control after delayed treatment of apple fruit with diphenylamine (DPA) and 1-methylcyclopropene (1-MCP)	Jung and Watkins (2008)
	'Granny Smith'	Effect of DPA and 1-MCP on chemical compounds related to superficial scald of 'Granny Smith' apples	Moggia et al. (2010)

	'Honeycrisp'	Prestorage conditioning and diphenylamine improve resistance to controlled-atmosphere-related Injury in 'Honeycrisp' apples	Contreras et al. (2014)
	'Granny Smith'	Investigating the metabolic changes after antioxidant treatment, temperature shifts and scald development	Leisso et al. (2014)
1-MCP	'McIntosh', 'Empire'	Responses of early, mid and late season apple cultivars to postharvest application of 1-methylcyclopropene (1-MCP) under air and controlled atmosphere storage conditions	Watkins et al. (2000)
	'Redchief Delicious'	Harvest maturity, storage temperature, and 1-MCP application frequency alter firmness retention and chlorophyll fluorescence of 'Redchief Delicious' apples	Mir et al. (1999)
	'Queen Cox', 'Bramley'	1-MCP improves storability of 'Queen Cox' and 'Bramley' apple fruit	Dauny and Joyce (2002)
	'Granny Smith'	Effect of heat or 1-methylcyclopropylene on antioxidative enzymes activities and antioxidants in apples in relation to superficial scald development	Shaham et al. (2003)
		Control of apple superficial scald and ripening-a comparison between 1-methylcyclopropene and diphenylamine postharvest treatments, initial low oxygen stress and ultra-low oxygen storage	Zanella (2003)
	'Empire'	Influence of 1- methylcyclopropene (1-MCP), diphenylamine (DPA), and CO_2 concentration during storage on 'Empire' apple quality	DeEll et al. (2005)
	'Cortland', 'Law Rome'	Ethylene and α-farnesene metabolism in green and red skin of three apple cultivars in response to 1-methylcyclopropene (1-MCP) Treatment	Tsantili et al. (2007)

	Superficial scald control after delayed treatment of apple fruit with diphenylamine (DPA) and 1-methylcyclopropene (1-MCP)	Jung and Watkins (2008)
'Granny Smith'	Effect of DPA and 1-MCP on chemical compounds related to superficial scald of Granny Smith apples	Moggia et al. (2010)
'Granny Smith'	Low oxygen and 1-MCP pretreatments delay superficial scald development by reducing reactive oxygen species (ROS) accumulation in stored 'Granny Smith' apples	Shabban-Amin et al. (2011)
'Cortland','Delicious'	Effects of repeated 1-methylcyclopropene (1-MCP) treatments on ripening and superficial scald of 'Cortland' and 'Delicious' apples	Lu et al. (2013)
'McIntosh, 'Spartan'	Investigating the effect of 1-methylcyclopropene treatments on fruit quality and storage disorders in apples	DeEll and Ehsani-Moghaddam (2013)
'Granny Smith'	Low oxygen pre-storage treatment is effective in reducing chilling injuries of deciduous fruit	Pesis et al. (2014)
	Use of the index of absorbance difference (IAD) as a tool for tailoring postharvest 1-MCP application to control apple superficial scald	Farneti et al. (2015)
	Target metabolite and gene transcription profiling during the development of superficial scald in apple (Malus x domestica Borkh)	Busatto et al. (2015)

Table 7

Superficial scald prediction models reported in literature.

Cultivar	Experimental parameter	Model	Reference
Cortland'	Hours below 10 °C	% scald=102+0.26h-0.01h^2 (r^2=0.76)	Barden and Bramlage (1994)
'Delicious'	Hours below 10 °C	% scald=158.3+0.90h+0.0003h^2 (r^2=0.69)	Barden and Bramlage (1994)
'Granny Smith'	Hours below 10 °C	% scald=0.0001x^3-0.283x^2+0.7655x+101.35 (r^2=0.97)	Thomai et al. (1998)
'Granny Smith'	night hours below 10 °C (N10) and 17°C (N17) before harvest	% scald=137.2+1.08(N10)-0.34(N17)	Lourens and Malherbe (1997)
	α-farnesene, days after full bloom (DAFB)	% scald=218.1-152.54(AF)-0.732(DAFB)	Lourens and Malherbe (1997)
'Starking'	Ratio of α-farnesene to conjugated trienes (CTs)	% scald=-5.76+173.71/x (r^2=0.93)	Zhang and Shu (2003)
'Ralls'	Ratio of α-farnesene to conjugated trienes (CTs)	% scald=133.34-17.04x+0.57x^2 (r^2=0.94)	Zhang and Shu (2003)
'Granny Smith'	Rate of conjugated trienes accumulation	% scald=$CTol_{258}$+[$CTol_{281}$]/2 (r^2=0.99)	Giné Bordonaba et al. (2013)

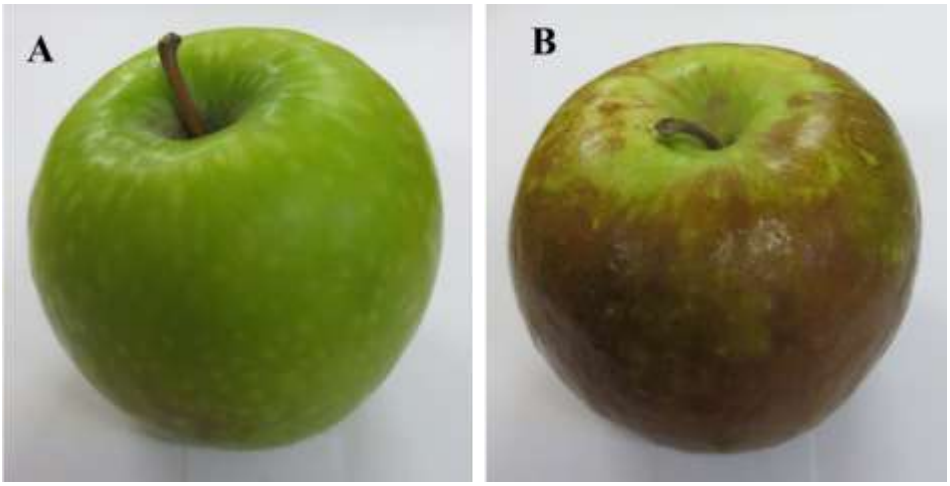

Figure 1: The difference between scald-free (A) and scalded (B) 'Granny Smith' apples.

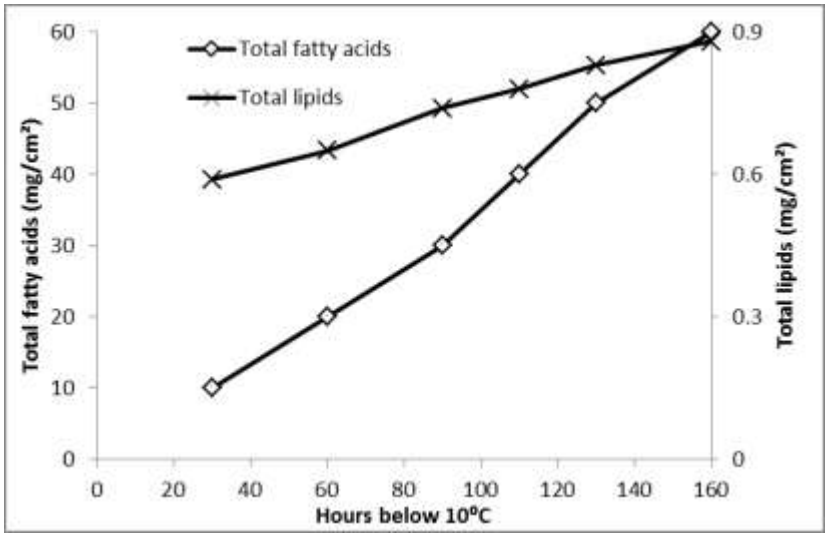

Figure 2: Effect of preharvest hours below 10 ^{0}C on total fatty acids and total lipids of 'Granny Smith' apple peel (Adapted from Thomai et al. 1998).

PAPER 2

Antioxidant contents and phytochemical properties of apples (cv. Granny Smith) at different harvest times

Abstract

Understanding the biochemical composition of fruit to be stored could be used as a tool for planning postharvest management to maintain quality and reduce losses of fresh fruit. This study evaluated the effects of harvesting fruit 7 days before (H1) and at optimal commercial harvest (H2) on the antioxidant contents and phytochemical properties of 'Granny Smith' apples. Both ascorbic acid content (345.6 vs 227.0 mg g^{-1}) and total antioxidant capacity (100.82 vs 82.37 µM g^{-1}) were significantly higher in H2 fruit while concentration of total phenolics was slightly higher in fruit harvested at H1 (25.34 vs 30.87 mg g^{-1}). Phenolic compounds including catechin, epicatechin and quercetin were significantly higher in fruit harvested at H1 compared to H2. These results showed that differences in antioxidant capacity and concentration of phytochemical contents are the major factors segregating fruit harvested before and at optimal commercial harvest.

Keywords: antioxidants, apples, harvest, physicochemical properties, vitamin C

1. Introduction

Apple is one of the frequently consumed fruit in the world. High contents of minerals, dietary fibre, and various biologically active compounds such as ascorbic acid, antioxidants and phenolics are nutritional characteristics of apples (Wu et al., 2007). Sensory qualities such as flavour, appearance and firmness are important in apple marketability (Lee et al., 2003; Lu et al., 2012). An array of postharvest challenges exist in apple industry. For example, supply of apple fruit in the market is often higher than demand. Various postharvest technologies are therefore used to reduce losses and wastage. Apples may be held for several months at low temperatures either at regular or controlled atmosphere storage. However, some cultivars are prone to storage physiological disorders such as superficial scald, core flush and bitter pit (Isidoro and Almeida 2006; Jemric et al., 2006; Sabban-Amin et al., 2011).

Climate, harvest maturity, storage conditions, and postharvest treatments are highly influential in postharvest performance of fruit (Fan et al. 1999; Wu et al. 2007). Kovač et al. (2010) found harvest maturity as a critical factor influencing flavour development and postharvest ripening. As reported by Veberic et al. (2005) and Silva et al. (2007) harvest maturity influences the sugar, phenolics and ascorbic acid content in apples. Generally, advanced fruit maturity reduces the propensity of postharvest physiological disorders such as superficial scald, greasiness and core flush (Ferguson et al., 1999). On the other hand, apples harvested at pre-optimal maturity may not fully ripen after storage and are highly susceptible to physiological disorders (Saevels et al., 2003). Studies have shown that early harvested apples are more susceptible to bruising (Opara, 2007). The major disadvantage of late harvested fruit is softness and greasiness after storage (Fan et al., 1999). Consequently, harvesting fruit at optimal maturity is essential to maintain postharvest quality. However, unforeseen circumstances such as high market prices and limited labour may necessitate early harvesting to meet market demand.

Ethylene gas is involved in aspects of ripening (Zanella, 2003), and ethylene production increases with fruit maturity and ripeness (Fan et al., 1999). However, the influence of ethylene in postharvest performance and storage potential of fruit is not clearly understood. Postharvest treatments such as controlled atmospheres (CA) and diphenylamine (DPA) for controlling superficial scald disorder suppress ethylene production (Whitaker 2000; Golding et al., 2001). Although ethylene manipulation is considered a ‘standard’ practise in maintaining the quality of stored produce, other physiological aspects play a prominent role in postharvest performance particularly in fruit from different maturities. The objective of this work was to assess the antioxidants contents and phytochemical properties of apples (cv. ‘Granny Smith’) harvested at 7 days before and at optimal commercial harvest.

2. Materials and methods

2.1 Fruit sample

Apple fruit (cv. ‘Granny Smith’) grown in two commercial orchards were used in this study. During commercial harvest period, fruit were hand-picked from Valley Green Farm in Grabouw (34.1212 S, 19.0235 E) and Erfdeel Farm in Ceres (33.3301 S, 19.6107 E), South Africa, at 165 and 172 days after full bloom (DAFB) (which are commonly considered in the fruit industry as pre- and optimal maturity periods, respectively), and transported to the

research laboratory at Agricultural Research Council, Stellenbosch and sorted to remove fruit with physical defects. The climatic conditions of the two ochards are shown in Table 1. Fruit firmness was measured using a Texture Analyser (Tensilon model UTM-4L, Tokyo Measuring Instruments Co., Ltd., Japan) with a 11.1mm compression probe. Firmness level on fruit from Valley Green orchard was 70.71 and 68.72 N for H1 and H2, respectively, whilst that of Erfdeel orchard was 79.83 and 74.43 N. Fruit starch content at the time of harvest was determined using iodine test method. Fruit from Erfdeel orchard had a 12.5% starch conversion when harvested at H1 and a 36.3% starch conversion when harvested at H2. The starch conversion for fruit harvested from Valley Green orchard was 12% and 35% for H1 and H2, respectively. Titratible Acidity (TA), expressed as malic acid, was measured at room temperature using titration to an endpoint of pH 8.2 with a Metrohm 862 compact titrosampler (Herisau, Switzerland). Fruit harvested at H1 had higher titratable acidity (TA) levels for both Valley Green (14.5mg $MAmL^{-1}$) and Erfdeel (15.6 mg $MAmL^{-1}$) orchard. The TA values for H2 fruit were 12.3 and 12.6 mg $MAmL^{-1}$ for Valley Green and Erfdeel orchard, respectively. The experiment was a 2x2 factorial design (factor A =Harvest time; factor B = Orchard). Fruit samples from each harvest maturity and orchard were divided into four groups, with three replicates containing 40 fruit each. Fruit of uniform size (±70 mm) and mass (±160 g) were used for measurements. For each treatment, 10 fruit per replicate were peeled under subdued light. The peel was immediately frozen with liquid nitrogen, freeze dried, pulverised and stored at -80^0C until use for extraction and measurement of total antioxidants, total phenolics and ascorbic acid.

2.2 Chemicals

Metaphosphoric acid, iodine, potassium iodide, methanol, 2,2-diphenyl-1-picrylhydrazyl, 2,6-dichlorophenolindophenol dye, 2,4,6-tripyridyl-s-triazine, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxyl acid, ascorbic acid, gallic acid, catechin, caffeic acid, epicatechin, quercetin, folin-ciocalteu, sodium carbonate, and ferric chloride were purchased from Sigma-Aldrich Co. (South Africa).

2.3 Phytochemical properties and antioxidant capacities

2.3.1 Sample preparation

Different extracts were prepared depending on analysis; 50% methanol for total phenolic and FRAP assays, 1% metaphosphoric acid for ascorbic assay and distilled water for

DPPH assay. One gram of pulverized apple peel was accurately weighed in centrifuge tubes using weighing balance, followed by the addition of 10 mL of the extraction solvents. The mixture was vortexed for 30 sec before being cold-sonicated for 10 min. The sample was thereafter centrifuged at 17764 g for 15 min at 4ºC to precipitate particulates. The extracts were carefully collected into test tubes and cold stored at 4ºC.

2.3.1.1 Ascorbic acid content

Ascorbic acid content was measured according to Fawole et al. (2012) with slight modifications. The mixture was vortexed for 30 sec before being ice-sonicated for 3 min, and thereafter centrifuged at 17764 g for 15 min at 4ºC. The extract (1 mL) was placed into a glass test tube and 9 mL of 2,6-dichlorophenolindophenol dye (0.0025%) was added. To ensure that only ascorbic acid is measured, the absorbance of the mixture was read at 515 nm within 30 min of incubation in dark environment (Barros et al., 2007). Ascorbic acid content was calculated using the calibration curve of authentic L-ascorbic acid (0.01–0.1 mg mL^{-1}), and the results were expressed as ascorbic acid equivalent (AAE) per grams dry matter (mg AAE mg g^{-1}DM).

2.3.1.2 Determination of total phenolics

Total phenolic (TP) content was determined according to Makkar et al. (2007) and with slight modifications by Fawole et al. (2012). Briefly, 450 µL of 50% methanol and 50 µL of extract were placed into glass test tubes. TP concentrations were determined spectrophotometically at 725 nm by adding Folin-Ciocalteu reagent to the juice sample after 10 min of incubation in the dark. Gallic acid was used as a standard and results were expressed as mean ± S.E (milligrams) of Gallic acid equivalents (mg GAE g^{-1}DM) of peel in triplicate samples.

2.3.1.3 Total antioxidant activities

2.3.1.3.1 Ferric reducing antioxidant power (FRAP) assay

Total antioxidant capacity was determined by the FRAP assay of Benzie and Strain (1996) with slight modifications. The FRAP assay measures the ability of antioxidants in the sample to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to the blue colored ferrous form (Fe^{2+}) that absorbs light at 593 nm (Khanizadeh et al. 2008). In triplicates, 150 µL of

the methanolic extract was mixed with 2.85 mL of FRAP reagent (300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution, 20 mM ferric chloride) and incubated in the dark for 30 min. Absorbance at 593 nm was measured using a spectrophotometer. TAC was expressed as mean ± S.E (micromoles) of Trolox equivalents per milligram of dry matter (µM TE mg^{-1}DM).

2.3.1.3.2 2,2-diphenyl-1- picrylhydrazyl (DPPH) assay

Peel extract sample was tested against a stable DPPH solution using the method of Wong et al. (2006) with some modifications using the distilled water extracts. Briefly, a 0.1 mM solution of DPPH in methanol was prepared. The initial absorbance of DPPH in methanol was measured at 515 nm and did not change throughout the assay period. In triplicates, 15 µLof the extract was mixed with methanol (735 µL) and subsequently with DPPH solution (750 µL, 01 mM). The change in absorbance at 515 nm was measured after 30 min incubation. Antioxidant activity based on the DPPH free radical scavenging ability was expressed as mean ± S.E (millimolar) of ascorbic acid equivalent per milligram of dry matter (mM AAEmg^{-1}DM).

2.3.1.4 HPLC-MS quantification of phenolic compounds

High-performance liquid chromatography-mass spectrometry (HPLC-MS) was used to determine the individual phenolic compounds in the methanolic peel extract. The LC-MS analysis of phenolics components in the apple peel extract was performed according to Mphahlele et al. (2014) using a Synapt G2 mass spectrometer UPLC system (Waters Corp., Milford, USA) connected to a photo diode array detector and a UPLC BEH C18 column (1.7 µm particle size, 2.1x100 mm, Waters Corp.) with injection volume of 3 µL at flow rate of 0.4 mL/min. Nitrogen was used as the desolvation gas, at a flow rate of 650 l/h and desolvation temperature of 275 °C. The gradient for the analysis of phenolic compounds started with 100% using 0.1% (v/v) formic acid (solvent A) and kept at 100% for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, 44% solvent B over 4 min and finally to 100% solvent B over 5 min. The column was subjected to 100% solvent B for an extra 2 min. The column was then re-equilibrated over 1 min to yield a total run time of 15 min. The electrospray ionization (ESI) probe was operated in the negative mode with the capillary voltage of 3 kV; and cone voltage of 15 V. Individual phenolic compounds were quantified by comparison with a multipoint calibration curve obtained from

the corresponding standards (catechin, epicatechin, caffeic acid and quercetin dihydrate) from Sigma Aldrich (South Africa).

3. Statistical analysis

Data was subjected to STATISTICA 11 (StatSoft, Inc. Oklahoma, USA) for two-factorial analysis of variance (factor A=harvest time; factor B= orchard) and Pearson's correlation. The results of all the studied variables are presented as mean (±S.E).

4. Results and discussion

4.1 Ascorbic acid content

Storage potential of fruit is influenced by many phytochemical compounds and antioxidants particularly ascorbic acid content in the peel. An inverse relationship between ascorbic acid and reactive oxygen species (ROS) exists, and it has considerable impact on postharvest performance. Ascorbic acid is the major antioxidant involved in ROS detoxification (Conklin, 2001). In fact, high levels of ascorbic acid are considered as important markers of fruit quality with potential of influencing postharvest life (Lu et al., 2012). In this study, harvest time affected ascorbic acid content (Table 2). Ascorbic acid content increased with advancing maturity for both orchards. Fruit harvested at H2 generally had higher ascorbic acid content. These results agree the pattern of ascorbic acid with advancing maturity for some apple cultivars as reported in the literature (Silva et al., 2007; Davey et al., 2007; Lu et al., 2012). In plants, ascorbic acid is the major antioxidant involved in stress alleviation (Conklin, 2001). High ascorbic acid content for H2 fruit could be the major reason for resistance of this fruit to postharvest physiological disorders.

Orchard location affected ascorbic acid content of fruit (Table 2). High ascorbic acid content was observed on fruit from Erfdeel orchard for both harvest times. For H2 fruit, ascorbic acid content of 256.1 and 345.6 mg g^{-1} DW was recorded for Valley Green and Erfdeel orchard, respectively. This variability might be attributed to climatic differences between the orchards (Olsson et al., 2004; Wang, 2006). High light intensity during the growing season is associated with high ascorbic acid accumulation (Lee and Kader, 2000). In this present study, fruit from orchard with high light intensity (Erfdeel; Table 1) had higher ascorbic acid content. Preharvest temperatures during growth and maturation have

pronounced influence on ascorbic acid content for some types of fruit. For instance, low ascorbic acid content in kiwifruit is linked with high temperatures during maturation (Richardson et al. 2004). Consequently, low ascorbic acid content on fruit from Valley Green orchard coincided with significantly high average temperatures.

4.2 Total phenolics

Phenolic compounds play a vital role in fruit resistance to postharvest stresses. High storage potential is commonly linked with high phenolic content of the fruit peel. Harvest date has enormous effect on phenolic content. Generally, optimally harvested fruit is characterized by high phenolic content (Kevers et al., 2011; Lu et al., 2012). Contrary, a highly significant phenolic content was found on H1 compared to H2 fruit (Table 2). This could be due to reduced 'polyphenol oxidase' gene expression during ripening process (Kim et al. 2011). Cultivar differences might influence the phenolic content-harvest maturity relationship (Lu et al. 2012). Schmitz-Eiberger and Matthes (2011) found no relationship between polyphenol contents and harvest maturity of 'Braeburn', 'Topaz', and 'Golden Delicious' apples.

Climatic conditions and cultural practices have a critical impact on total phenolic content (Wang, 2006; Babbar et al., 2011). In this study, orchard and harvest maturity had a significant interaction ($p \leq 0.01$). However, fruit from Valley Green orchard appleared to have higher phenolic content compared to Erfdeel orchard. The total phenolic content on fruit from Valley Green orchard ranged from 26.98 to 30.87 mg g^{-1} whilst that of Erfdeel orchard ranged from 25.34 to 29.63 mg g^{-1}. This result contradicts previous findings by other researchers. For instance, Jakopic et al. (2009) found high light intensity to increase total phenolic content in 'Fuji' apples. Moreover, high total phenolic content on 'Gala' apple fruit exposed to high light intensity has been reported (González-Talice et al., 2013). However, this could not be confirmed in this present study despite the distinct climatic differences between the orchards. In fact, low phenolic content was recorded on fruit from Ceres production area despite its high light intensity (Table 1). The relationship between light intensity and total phenolic content might vary among apple cultivars.

Air temperature during growing season plays a major role in phenolic composition of the fruit with low mean temperatures being associated with increased total phenolic concentrations in apples (cv. 'Cox's Orange', 'Granny Smith', 'Pacific Queen') (McGhie et

al., 2005). However, in this study, 'Granny Smith' apples from growing locations with higher temperatures had higher phenolic content. It is possible that cultivars respond differently to the accumulation of phenolic compounds under different growing environment conditions. However, this hypothesis needs to be tested further for wide range of cultivars and other types of fruit.

4.3 Total antioxidant capacity

The effect of harvest time on antioxidant capacity was observed (Table 2). Antioxidant capacity measured by FRAP assay significantly varied (p<0.01) between harvest times on fruit from Erfdeel orchard but not for fruit from Valley Green orchard. Antioxidant capacity followed an increasing pattern with advancing maturity irrespective of orchard. The radical antioxidant power measured by DPPH assay varied between harvest times for both orchards, with fruit at H2 having significantly higher antioxidant capacity. This result is in agreement with the increasing antioxidant capacity with advancing maturity reported by previous researchers for 'Granny Smith' (Vasilakakis and Manseka, 1993) and 'Golden Delicious' apples (Torres et al., 2003).

Based on these findings, the high antioxidant capacity of H2 fruit could be an indication that this could offer protection of the fruit from postharvest abiotic and biotic stresses; however, further studies are warranted to test this hypothesis. Previous research has shown that postharvest pathological and physiological disorders are highly pronounced on low antioxidant containing fruit (Davey et al., 2007). Generally, pre-optimal harvested fruit is highly susceptible to postharvest stress. However, increasing resistance is linked with increasing harvest maturity (Torres et al., 2003). Preharvest exposure to high temperatures can influence the phytochemical contents and quality attributes of apple fruit. Despite their differences in temperatures, orchards showed no significant differences in antioxidant capacity. These results contradict those reported elsewhere that climate impacts on fruit metabolite levels and antioxidant capacity (Lee and Kader, 2000; Wang, 2006; Davey et al., 2007).

4.4 Phenolic compounds

Individual phenolic compounds including catechin, epicatechin and quercetin were identified. However, no caffeic acid was detected in the samples. Phenolic compounds

significantly varied (p<0.01) between harvest times. Catechin levels decreased with advancing maturity for both orchards (Figure 1). Fruit harvested at H1 generally had higher catechin concentration than H2 harvested fruit. Changes in catechin concentration during apple maturation have previously been reported. Mosel and Herrmann (1974) and Amiot et al. (1992) reported a sharp decline of catechin concentration with advancing maturity in 'Golden Delicious' and 'Granny Smith' apples. In contrast, Alonso-Salces et al. (2005) found catechin concentration to increase with maturity. Catechin concentration was thereafter used as a chemical marker to characterise apples according to their maturity (Alonso-Salces et al., 2005).

Epicatechin varied between harvest times for both orchards, with fruit at H1 having significantly higher epicatechin concentration (Figure 2). Similar results were observed by Burda et al. (1990) in 'Rhode Island Greening' apple peel. Harvest time did not significantly affect (p>0.05) quercetin content in both orchards (Figure 3). These results are contrary to Alonso-Salces et al. (2005) who reported a gradual increase of quercetin concentration with advancing maturity in 12 apple cultivars apples. The reduction of phenolic compounds could be linked to total phenolics which also declined with advancing maturity. In fact, correlation was found between total phenolics and catechin (r^2=0.658) and between catechin and epicatechin (r^2=0.687) phenolics. Previous studies have shown that the expression of polyphenol oxidase, a key gene involved in the synthesis of phenolics, declines during ripening process (Kim et al., 2011).

Previous studies have reported growing region and cultural practices as determinants of antioxidants and phenolic compounds concentration in apples. For instance, McGhie et al. (2005) reported significantly different phenolic concentration on 'Granny Smith', 'Pink Lady', and 'Pacific Rose' apples from three growing regions. In this study, orchard had insignificant effect (p>0.05) in all the phenolic compounds investigated.

4.5 Treatment interactions

Harvest time x orchard interaction was significant for physicochemical properties and antioxidants contents. For ascorbic acid and antioxidant content as measured by FRAP assay, an interaction between harvest time and orchard was detected (p<0.001), with difference between fruit from orchards occurring at both harvest time. Total phenolic content was also affected by harvest time x orchard interaction with contents decreasing with advancing

maturity. However, DPPH assay showed a non-significant (p=0.148) harvest time x orchard interaction in antioxidant content. Harvest time and orchard interaction was insignificant for phenolic compounds. These results suggest that due to strong harvest time x orchard interaction, total phenolics and vitamin C might not be reliable biochemical parameters for predicting storage potential of fruit.

5. Conclusion

This study showed that apple fruit harvested before and at commercial harvest varied significantly in physicochemical and biochemical composition. Total antioxidants, total phenolics and ascorbic acid proved to be major factors in discriminating fruit maturity levels. Thus, high storage potential of optimally harvested fruit as previously reported in literature could be linked to high antioxidant pool. This study also highlighted the possible role of climatic conditions during fruit growth and maturation postharvest performance of apples (cv. 'Granny Smith') climatic conditions. Favourable climatic conditions, particularly light intensity, seem to play an important role in enhancing the phytochemical contents and antioxidant capacity of fruit.

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Table 1

Climatic conditions at the two apple growing locations in the Western Cape, South Africa (ARC-ISCW). Values in the same column with the same superscript letter are not significantly different ($p < 0.05$; Duncan's multiple range test).

Production Area	Altitude (m)	Rainfall (mm)	Avera. Temp (^{0}C)	Light Intensity (mj m^{-2})	Daily Positive Chilling Units
Grabouw	300	867.7a	15.8a	16.2a	137.6b
Ceres	947	640.5b	14.2b	18.3b	211.5a

Table 2

Antioxidant and phytochemical contents of 'Granny Smith' apples harvested before (H1) and at commercial harvest (H2) from two orchards in the Western Cape Province, South Africa. Data are means ± SE. Values in the same column with the same superscript letter are not significantly different ($p < 0.05$; Duncan's multiple range test).

Orchard	Harvest	Ascorbic acid (mg AAE g^{-1})	Total Phenolics (mg GAE g^{-1})	FRAP (µMTE g^{-1})	DPPH (mM AAE g^{-1})
Valley Green	H1	227.00±49.00d	30.87±1.28a	90.09±1.48b	1.33±0.04c
	H2	256.10±49.00c	26.98±1.28bc	92.08±1.48b	1.82±0.04a
Erfdeel	H1	296.30±49.00b	29.63±1.28b	82.37±1.48c	1.51±0.04b
	H2	345.60±49.00a	25.34±1.28c	100.82±1.48a	1.78±0.04a
Significance	Harvest time (A)	<0.001	<0.001	<0.001	<0.001
	Orchard (B)	<0.001	<0.001	0.733	0.146
	A*B	<0.001	<0.001	<0.001	0.148

Values (mean ± S.E.) in the same column followed by different letter(s) are significantly different ($p<0.05$) according to Duncan's multiple range test

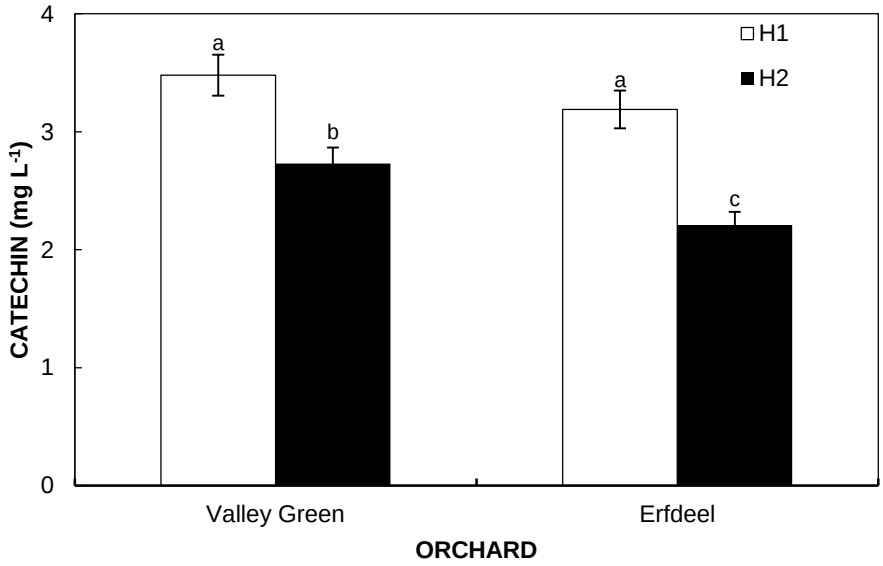

Figure 1: Catechin concentration of 'Granny Smith' apples harvested before (H1) and at commercial harvest (H2) from two orchards in the Western Cape Province, South Africa. Bars with the same letter are not significantly different ($p > 0.05$)

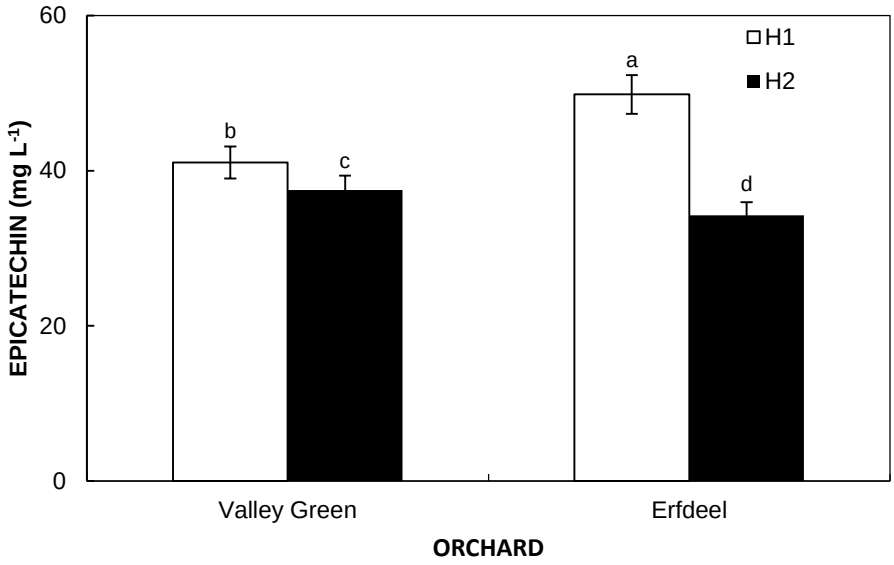

Figure 2: Epicatechin concentration of 'Granny Smith' apples harvested before (H1) and at commercial harvest (H2) from two orchards in the Western Cape Province, South Africa. Bars with the same letter are not significantly different ($p > 0.05$)

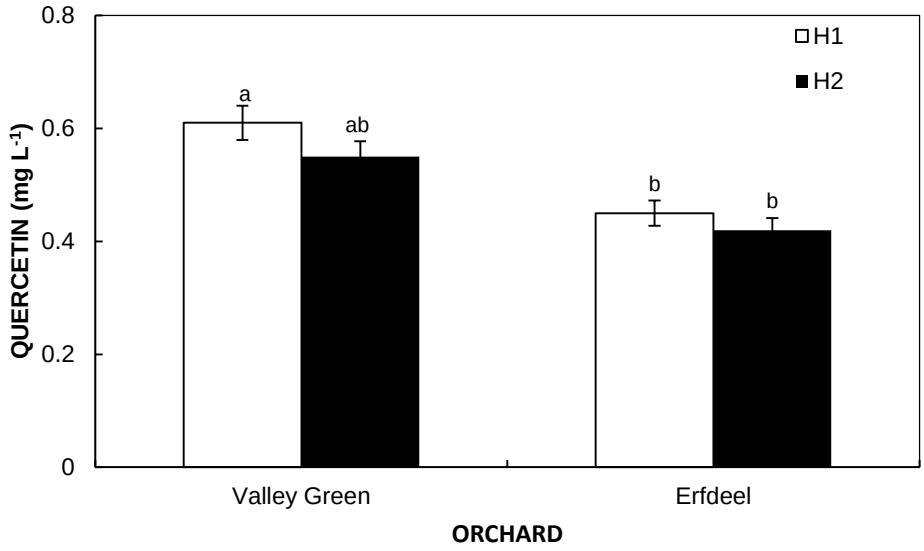

Figure 3: Quercetin concentration of 'Granny Smith' apples harvested before (H1) and at commercial harvest (H2) from two orchards in the Western Cape Province, South Africa. Bars with the same letter are not significantly different ($p > 0.05$)

PAPER 3

Classification of 'Granny Smith' apples with different levels of superficial scald severity based on metabolomics and discriminant analysis

Abstract

To study the metabolomic changes in 'Granny Smith' apples with different severities of superficial scald, fruit were stored in normal refrigerated air (0°C, 95% RH) for 12 weeks followed by 7 d shelf-life under room conditions (20°C, 65% RH). Fruit were graded to five groups based on scald severity and analysed for ethylene, α-farnesene and 6-methyl-5-hepten-2-one (MHO) levels. Reactive oxygen species (ROS) were measured by confocal laser-scanning microscopy on apple peel treated with fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate. Ethylene production rate, α-farnesene and MHO contents and ROS intensity increased with increasing scald severity but declined in severely scalded fruit. Malondialdehyde (MDA) concentration in fruit peel, a measure of membrane damage, increased linearly (R=0.891) with increase in scald severity. Discriminant analysis was used to classify fruit by scald severity on the basis of metabolites accumulated. The stepwise model indicated that three attributes (ROS, ethylene production and MDA) contributed significantly ($R^2 \geq 0.5$) to the separation of the five scald severity indexes, with ROS having the highest contribution (partial $R^2 = 0.961$; $p < 0.0001$), followed by ethylene ($R^2 = 0.718$; $p < 0.0001$) and MDA ($R^2 = 0.578$; $p < 0.0001$).

Keywords: Scald severity, ethylene, α-farnesene, 6-methyl-5-hepten-2-one, reactive oxygen species, lipid peroxidation

1. Introduction

Superficial scald is the major physiological disorder that develops after long term cold storage of apples and is associated with the disruption of tissues immediately beneath the epidermis of the fruit, with tissue browning not extending to the mesocarp of pulp (Bain, 1956; Sabban-Amin et al., 2011; Lurie and Watkins, 2012). Array of factors such as cultivar, seasonality, maturity, and duration of cold storage significantly influence scald development (Rao et al., 1998; Watkins et al., 2000; Ahn et al., 2007). Some apple cultivars such as 'Granny Smith' and 'Law Rome' are more susceptible (Watkins et al., 1995; Pechous and Whitaker 2004; Jemric et al., 2006; Sabban-Amin et al., 2011) whilst cultivars such as

'Golden Delicious' and 'Royal Gala' are resistant (Ingle 2001; Zanella et al., 2008; Beuning et al., 2010).

In particular, fruit maturity plays an important role in scald susceptibility, with scald incidence declining with advancing fruit maturity (Erkan and Perkmezci, 2004). There are physiological changes preceding superficial scald development. Generation of reactive oxygen species or oxidative stress coupled with α-farnesene synthesis is one of the primary events leading to scald symptoms (Rudell et al., 2009). Moreover, conjugated trienols (CT) and 6-methyl-5-hepten-2-one (MHO), both oxidised products of α-farnesene, increase with scald incidence (Mir et al., 1999; Rowan et al., 2001; Moggia et al., 2010). Although concerted research efforts have been made to understand metabolomic events leading to scald development, the relationship between scald severity and accumulation of metabolites linked to scald development remains unclear. The objective of this research work was to investigate the relationship between scald severity and metabolomic changes in 'Granny Smith' apples.

2. Materials and methods

2.1 Fruit source and treatments

Apple fruit (cv. Granny Smith) grown in a commercial orchard were hand-picked from Valley Green Farm in Grabouw (34° 12'12" S, 19° 02'35" E), South Africa, at optimal maturity, and transported to the Research Laboratory at Agricultural Research Council, Stellenbosch and sorted to remove fruit with physical defects. The experiment was laid in a completely randomised design. Uniformly sized fruit with diameter of 70±2 mm and mass of 160±5 g were randomly selected to provide three replications of 100 fruit each. Fruit were stored in regular atmosphere (RA) at 0°C (95% RH) for 12 weeks followed by 7 days shelf-life at normal room conditions (20°C, 65% RH). At the end of shelf life, fruit were individually assessed and rated for scald severity based on the percentage of the surface area affected and sorted into five groups based on as follows: 0 = no scald, 1 = 1-25% (slight), 2 = 26-50% (moderate), 3 = 51-75% (high), and 4 = 76-100% (very high) (Fig. 1). For each level of scald severity, six replicates with five fruit per replicate were used for analysis.

2.2 Ethylene production

Ethylene production was measured as described by Oz (2011) with slight modifications. Briefly, each fruit was weighed using a Mettler Toledo digital balance (±0.01g), and thereafter enclosed in 1 L airtight jar for 1 h at 20°C. Infrared ethylene analyser (ICA56 ppm) was used for measurement and the results were expressed as $\mu L C_2H_4 kg^{-1} h^{-1}$.

2.3 Headspace volatile analysis

Fresh peel (5 g) was weighed into a 20 mL solid phase microextraction (SMPE) glass vials. 10 µL of 3-octanol internal standard was added, and the vials were sealed. Vial headspace was analysed according to Mayuoni-Kirshinbaum et al. (2012) and Caleb et al. (2013). The vials were equilibrated for 10 min at 50 °C in the CTC autosampler incubator. After equilibration, a 50/30 µm divinylbenzene/-carboxen/-polydimethylsiloxane coated fibre was then exposed to the sample headspace for 20 min at 50 °C. After extraction, the trapped volatile compounds from the fibre coating were desorbed for 2 minutes in the injection port of the gas chromatograph operated in a splitless mode. The temperature was maintained at 250ºC for the injection. The fibre was cleaned after each sample heating for 10 min in the fibre conditioned station maintained at 270ºC. Chromatographic separation of the extracted volatiles was performed on a Agilent 6890N (Agilent, Palo Alto, CA) connected through a transfer line to a Agilent 5975B MS (Agilent, Palo Alto, CA) mass spectrometer detector. The GC–MS system was equipped with a polar DB-FFAP column from J&W (part number 122-3263) with the following dimensions: (60 m length; 250 µm internal diameter; and 0.5 µm film thickness). Helium was used as carrier gas at a flow rate of 1.3 mL min^{-1}. The oven temperature program was as follows: initial temperature of 40 °C for 5 minutes; then ramped at 5 °C min^{-1} up to a final temperature of 230 °C with a final hold time of 6 minutes. The ion source and quadropole were maintained at 240 °C and 150 °C, respectively. The transfer line temperature was maintained at 280 °C. Alpha-farnesene and MHO were identified by a library search and quantified using abundance characteristic ion 93 and 108, respectively. Generally, α-farnesene gave a single peak at 22.2 min while MHO gave a peak at 16.6 min. A reading of 10^4 in abundance was defined as one unit and expressed as U g^{-1} (Ju and Curry, 2000).

2.4 Confocal microscopic analyses of ROS production

Reactive oxygen species were determined following a method described by Macarisin et al. (2007) and Sabban-Amin et al. (2011). The fluorescent probe 2,7-dichlorodihydrofluorescein diacetate in which dichlorodihydrofluorescein (DCF) fluorescence measurement quantifies general oxidative stress was used. 2,7-dichlorodihydrofluorescein enters cells in the diacetate form (H_2DCF-DA), and the acetate form (H_2DCF) is hydrolyzed by intracellular esterases and then reacts with oxidants, resulting in the highly fluorescent DCF. Acetate detects a broad range of oxidizing molecules rather than a single ROS form, and it is efficient in localizing ROS within plant cells (Joo et al., 2005). Immediately before microscopic analysis, slices of apple peel were cut from fruit and immediately immersed in a small Petri dish containing 10 mL of 10.0 µM H_2DCF-DA in loading buffer (50 mM MES buffer, pH 6.5). The H_2DCF-DA was freshly prepared from a 20 mM stock solution in dimethyl sulfoxide (DMSO). To prevent light-inducible oxidation, the slices were kept in the dark for 10 min and were thereafter transferred to a new Petri dish containing loading buffer to wash off excess dye. Model IX 81 inverted confocal laser-scanning microscope (FLUOVIEW 500, Olympus, Japan) equipped with a 488 nm argonion laser was used for sample examination and image acquisition. The fluorescent probe was excited with a 488 nm laser beam and the emission was collected through a BA 515–525 filter. For autofluorescence, a BA 660 IF emission filter was used. Magnification was increased by focusing the scanning laser beam onto a smaller area of the tissue. The transmitted-light images were obtained with Nomarski differential interference contrast (DIC) optics. The relative intensity of the fluorescence signal was estimated by calculating average pixel intensity from each successive focal plane of the apple peel slice, in 5 µm steps, with MICA software (Multi-Image Analysis, CytoView, Israel). The value of fluorescence intensity presented is the mean (± standard error (SE)) of five different slices.

2.5 Lipid peroxidation

Malondialdehyde (MDA) is regarded to be a suitable biomarker for lipid peroxidation caused by ROS which is the major cause of membrane damage in plant tissues (Katsuhara et al., 2005; Lu et al., 2014). MDA was measured by the method described by Dhindsa et al. (1981) and Siboza et al. (2013) with slight modifications. Freeze dried and pulverised apple peel (0.1g) was homogenised with 10 mL of ice cold 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 rpm for 15 min at 4ºC to precipitate particulates. A 1

mL aliquot of the supernatant was thoroughly mixed with 4 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was incubated at 95°C for 30 min and thereafter quickly cooled in an ice bath. After centrifugation at 10000 for 15 min at 4°C, the absorbance of the supernatant was read at 532 nm and corrected for nonspecific absorbance at 600 nm using UV-Visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). The concentration of MDA was calculated using an extinction coefficient (∈) of 155 mM^{-1} cm^{-1}.

2.6 Statistical analysis

Data was subjected to Statistica 11 (StatSoft Inc. Oklahoma, USA) for analysis of variance (ANOVA) according to Duncan's multiple range test. Graphical data presentations were performed using GraphPad Prism software, version 6 (GraphPad Software, Inc. San Diego, USA). Discriminant analysis (DA) was performed using XLStat, version 7.5.2 (Addinsoft, New York, USA). By analysing treatment scores or component loading values derived from discriminant analysis, associations between treatment and metabolic components were be uncovered.

3. Results and Discussion

3.1 Ethylene

Ethylene production has an influence on physiological changes associated with superficial scald (Ingle, 2001). Several reports have shown that ethylene is involved in regulating α-farnesene, a key volatile involved in the induction of superficial scald in apples (Mir et al., 1999; Ju and Curry, 2000; Pechous et al., 2005). In this study, ethylene production increased gradually as scald severity increased from none (0) to slight (2), and declined as scald severity progressed (Fig. 2). Reduced ethylene production with increasing scald severity could be an indication of its completed role in scald etiology. This observation is in agreement with previous findings by Mir and Beaudry (1999), who demonstrated that ethylene production increases with scald-development but reduces in severely scalded fruit. A similar trend has also been reported for 'Bartlett' pears stored at -1°C for 24 weeks (Ekman et al., 2004; Whitaker et al., 2009).

Sabban-Amin et al. (2011) found that ethylene production was strongly associated with MdACS gene expression. However, MdACS expression declined with extended storage

period. Reduced ethylene production in severely scald fruit could therefore be linked to reduced MdACS gene expression. Moreover, the putative role of ethylene could be linked to the early stages of scald development, and hence the low ethylene production in severely scalded fruit.

3.2 Headspace volatile analysis

Scald development has been strongly associated with α-farnesene accumulation in apple peel (Pesis et al., 2009). In this study, headspace levels of α-farnesene increased with scald severity (Table 1) but declined thereafter when fruit became highly scalded. This result is in agreement with previous findings which demonstrated that α-farnesene increased with scald severity in 'Law Rome' apples (Watkins et al., 2000) and 'Granny Smith' apples (Shaham et al., 2003; Zubini et al., 2007) stored at 0°C for up to 26 weeks. As noted in this study, Zubini et al. (2007) also reported a decline in α-farnesene levels in severely scalded fruit.

Gapper et al. (2006) and Sabban-Amin et al. (2011) found α-farnesene production to directly correspond to the expression of α-farnesene synthase gene *AFS1* in 'd' Anjou' pears and 'Granny Smith' apples, respectively. Moreover, the levels of α-farnesene and *AFS1* transcript increased with storage time and scald incidence, and suddenly declined for the remainder of storage. The decline in α-farnesene content of severely scalded fruit could therefore be linked to reduced *AFS1* expression during senescence. The decline in ethylene production coincided with a decrease in α-farnesene content. Interestingly, Gapper et al. (2006) reported a similar trend for 'd' Anjou' pears and concluded that *AFS1* transcript is mediated by ethylene.

MHO (a product of α-farnesene oxidation) was significantly lower in fruit with no scald and increased with the onset of scalding and up moderate severity however, after severity level 2 (26-50%), there was a decline in MHO content (Table 1). Our findings are corroborated by those reported in 'Granny Smith' apples by several researchers (Fan et al., 1999; Mir et al., 1999; Wang and Dilley, 2000) who demonstrated an initial increase in MHO content with onset of scald and a subsequent decline as scald incidence and severity increased. It is worth noting that the reduction in both α-farnesene and MHO levels coincided with a decline in ethylene production. These results suggest that both the production and oxidation of α-farnesene may require ethylene action. In fact, previous studies focused on

reducing superficial scald in apples reported a reduced α-farnesene and MHO production after 1-MCP treatment, and consequently low scald incidence (Ghahramani and Scott, 1998; Fan and Mattheis, 1999; Shaham et al., 2003; Gapper et al., 2006; Jung and Watkins, 2008). However, Mir et al. (1999) indicated that the temporary relationship between scald severity and MHO may indicate that MHO is not directly involved in scald development. Contrary to the prevailing hypothesis linking MHO and superficial scald, Rupasinghe et al. (2000) reported that methyl heptenol (MHOL) in 'Delicious' apples stored at 0°C for 17 weeks was 60% and 20% higher in scald-developing and severely scalded tissues, respectively. Other research findings have concluded that MHO production rather than its presence, is the important aspect involved in scald appearance (Ju and Curry, 2002; Lurie and Watkins, 2012). Similar to the direct relationship between *MsAFS1* expression and α-farnesene production (Ju and Curry, 2000; Lurie et al., 2005; Gapper et al., 2006), there could be a gene responsible for MHO production which is probably less expressed in severely scalded fruit. Further research is warranted to evaluate this possibility.

3.3 ROS detection and quantification

Low storage temperatures trigger plant tissues to produce reactive oxygen species (ROS) which are the by-products of electron flow disruption in the mitochondria (Purvis et al., 1995; Pinhero et al., 1997) resulting in physiological disorders such as chilling injury (Lyons, 1973; Sala, 1998) and superficial scald (Watkins, 1995; Sabban-Amin et al., 2011). Physiologically, ROS cause the oxidative stress that consequently results in imbalances in metabolism, high respiration rate, reduced ability of biological systems to detoxify toxic metabolites (Lyons, 1973). The fluorescence appearing during cold storage and shelf-life was quantified as fluorescence units related to ROS levels (Sabban-Amin et al., 2011; Pesis et al., 2012). In the current study, fluorescence intensity significantly increased with scald severity (Fig. 4A-E). Similarly, ROS levels increased with scald severity (Fig. 3); however, low fluorescence and ROS levels were detected in severely scalded fruit (Fig. 4E).

These results have demonstrated that ROS possible plays a role in superficial scald development in 'Granny Smith' apples. This observation is consistent with previous findings that ROS are involved in scald etiology. For instance, Rao et al. (1998) found scald incidence to be related to ROS levels in hybrid 'White Angel x Rome Beauty' apple stored for 16 weeks at 0.5°C. Moreover, hydrogen peroxide (H_2O_2) was reported by Zubini et al. (2007) to

increase with scald incidence and severity in 'Granny Smith' apples. Recently, Lu et al. (2014) also reported scald severity to be highly dependent on the accumulation of H_2O_2 concentration in 'Fuji' apples stored at 0°C for 28 weeks. Our study shows that the accumulation of ROS is linked to scald severity, and the low ROS levels in high to severely scalded fruit could be linked to reduced reactivity of oxygen species, and hence the corresponding reductions in metabolites implicated in scald development and severity such as α-farnesene and MHO.

3.4 Lipid peroxidation

Unless metabolised, ROS cause lipid peroxidation and eventual symptoms of damage in plant tissues. Peroxidation of membrane lipids is an indication of membrane damage and electrolyte leakage under cold stress (Katsuhara et al., 2005). Lipid peroxidation leads to membrane damage, and consequently chilling injury symptoms (Lyons, 1973). Membrane damage is the primary metabolism disorder preceding superficial scald in apples (Rao et al., 1998). In this study, scald severity had a significant effect on lipid peroxidation expressed as malondialdehyde (MDA) concentration (Fig. 5). The MDA was significantly lower in fruit with no scald. However, lipid peroxidation gradually increased with scald severity. This result is in agreement with previous findings showing the accumulation of MDA in scalded apples. For instance, Rao et al. (1998) reported that lipid peroxidation increases with storage time and consequently scald severity in 'White Angel x Rome Beauty' apple. Lu et al. (2014) also noted that MDA content increases with scald incidence and severity in 'Fuji' apples stored at 0°C for 28 weeks.

Moggia et al. (2010) reported increased membrane integrity in severely scalded 'Granny Smith' apples for 6 months at 0°C. Thomai et al. (1998) also demonstrated that membrane damage increases with scald incidence and severity. The low lipid peroxidation in fruit with no scald shows the relationship between scald incidence and membrane damage. Moreover, the continuous increase in lipid peroxidation indicates that superficial scald is not only a change in symptoms but also an accumulative damage (Lu et al., 2011).

3.5 Discriminant analysis

Outcome of discriminant analysis of scald severity index and metabolic attributes is presented in Fig. 6. Confusion matrix showing the correct and incorrect predictions made by

the model are presented in Table 2. The confusion matrix indicated 96.76% accuracy in classifying the five scald severity classes. Four indexes were particularly well discriminated with 100% accuracy; however, 16.67% confusion appeared in severely scalded fruit.

The stepwise model indicated that three attributes, namely; ROS, ethylene production and MDA contributed significantly ($R^2 \geq 0.5$) to the separation of the five scald severity indexes (Table 3). Amongst the contributors, ROS had the highest significant ($p<0.0001$) contribution with $R^2 = 0.961$, suggesting that ROS could indeed be strongly linked to scald severity in 'Granny Smith' apples. This result is in agreement with Rao et al. (1998), who indicated that although scald development mechanism is yet to be fully understood, the contribution of reactive oxygen species (ROS) maybe related to the disorder.

4. Conclusion

This study has demonstrated that scald severity is not directly related to some scald-associated metabolites in 'Granny Smith' apples. While increases in ethylene production, α-farnesene and MHO corresponded with the onset and progression of scald severity, this relationship did not hold in high to severely affected fruit. However, the accumulation of ROS leading to loss of membrane integrity corresponded strongly to the level of scald severity. These findings suggest that scald resistance of 'Granny Smith' apples might be enhanced by improving fruit ability to metabolise ROS during storage.

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Table 1

Headspace accumulation of α-farnesene and MHO in 'Granny Smith' apples with different levels of scald severity.

Superficial scald index	α-farnesene ($U\ g^{-1}$)	MHO ($U\ g^{-1}$)
0	6612.09 c*	56.18 c
1	11216.05 b	90.17 b
2	15286.12 a	132.04 a
3	11716.10 ab	110.01 b
4	8940.15 bc	84.13 bc

*Mean values followed by the different letter differ significantly according to Duncan's multiple range test ($p<0.05$).

Table 2

Confusion matrix of 'Granny Smith' apples with different levels of superficial scald severity

Superficial Scald Index	0	1	2	3	4	Total	% correct
0	6	0	0	0	0	6	100.00
1	0	6	0	0	0	6	100.00
2	0	0	6	0	0	6	100.00
3	0	0	0	6	0	6	100.00
4	1	0	0	0	5	6	83.33
Total	7	6	6	6	5	30	96.67%

Table 3

Summary of variable selection using stepwise analysis showing attributes that contribute most to superficial scald severity of 'Granny Smith' apples

Variable	Status	Partial R^2	F statistic	Pr > F
ROS	IN	0.961	153.506	<0.0001
Ethylene	IN	0.718	15.306	<0.0001
MDA	IN	0.578	7.887	<0.0001

ROS, reactive oxygen species; MDA, malondialdehyde.

Partial R^2 - determination coefficient; F statistic - F ratio test; Pr > F - p value at significance level of 0.05.

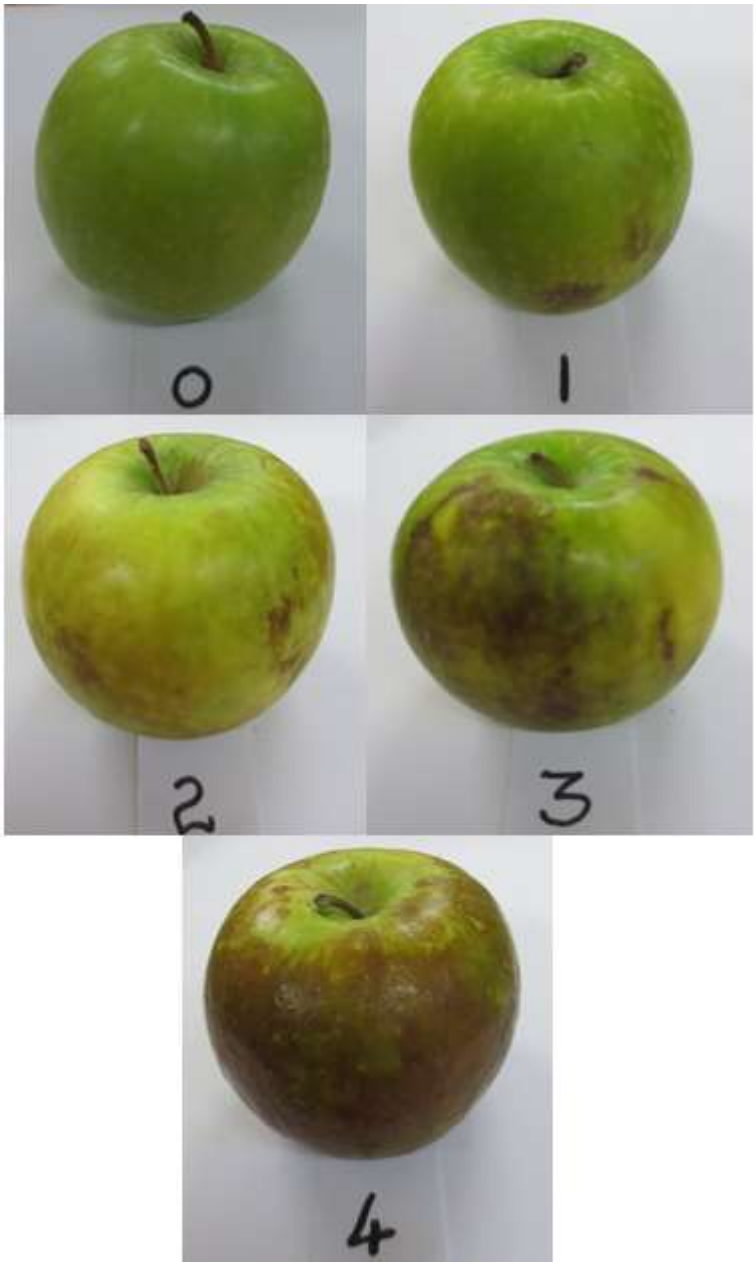

Fig. 1 'Granny Smith' apples with different superficial scald severity: 0, no scald; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%.

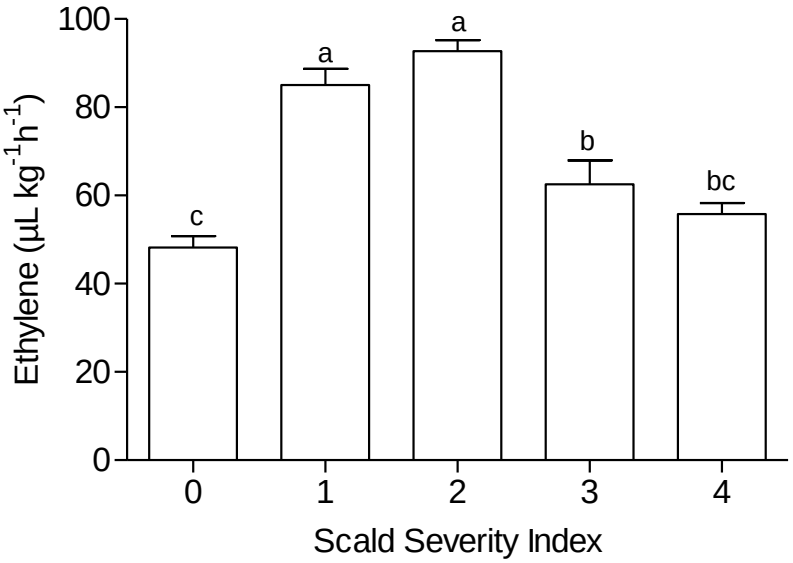

Fig. 2 Ethylene production in 'Granny Smith' apples with different scald severity. Data points with different letter(s) differ significantly according to Duncan's multiple range test ($p<0.05$).

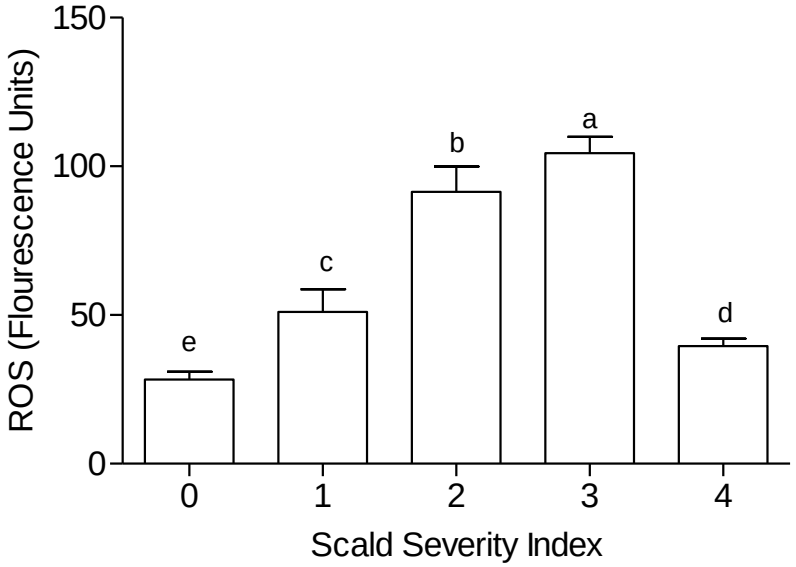

Fig. 3 Production of reactive oxygen species (ROS), as quantified by relative intensity of dichlorodihydrofluorescein diacetate (DCF) fluorescence in peel slices of 'Granny Smith' apples with different scald severity. Data points with different letter(s) differ significantly according to Duncan's multiple range test ($p<0.05$).

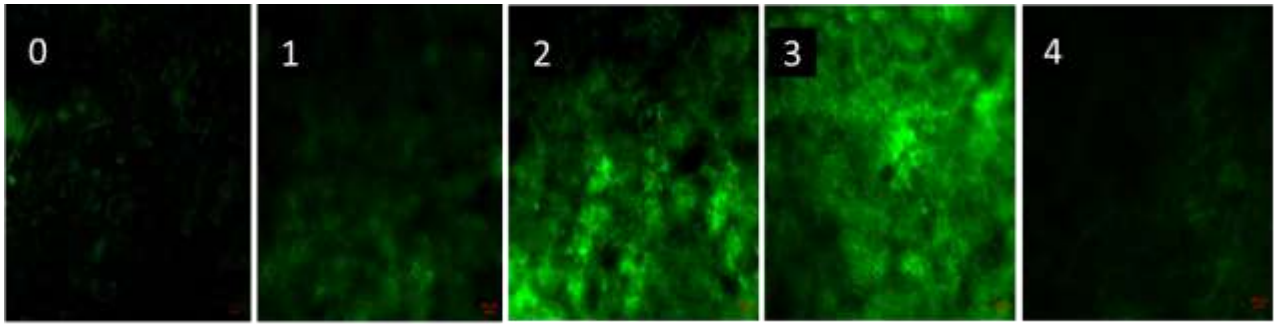

Fig. 4 Confocal laser-scanning fluorescence images of 'Granny Smith' apple peel slices. Superficial scald index 0, no scald; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-100%.

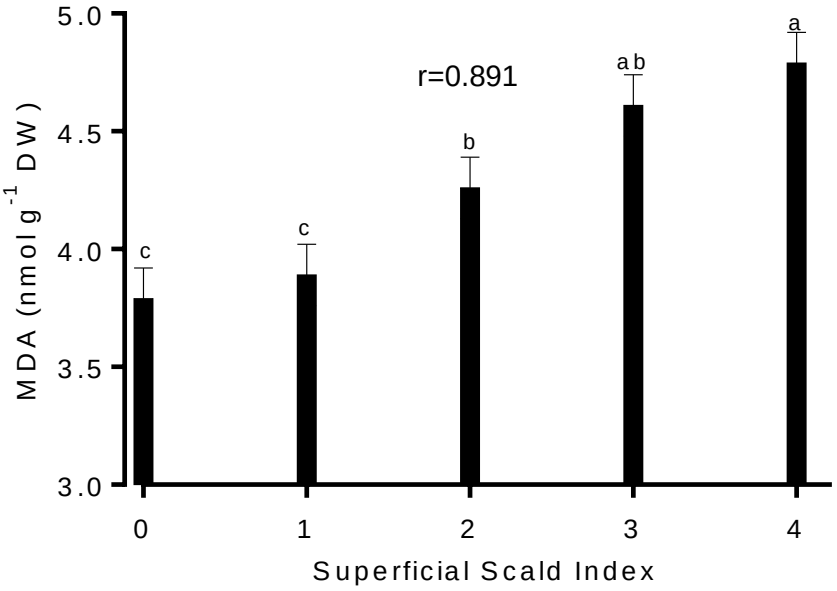

Fig. 5 Changes in malondialdehyde (MDA) concentration of 'Granny Smith' apple peel slices as influenced by scald severity. Bars with different letter(s) differ significantly according to Duncan's multiple range test ($p<0.05$).

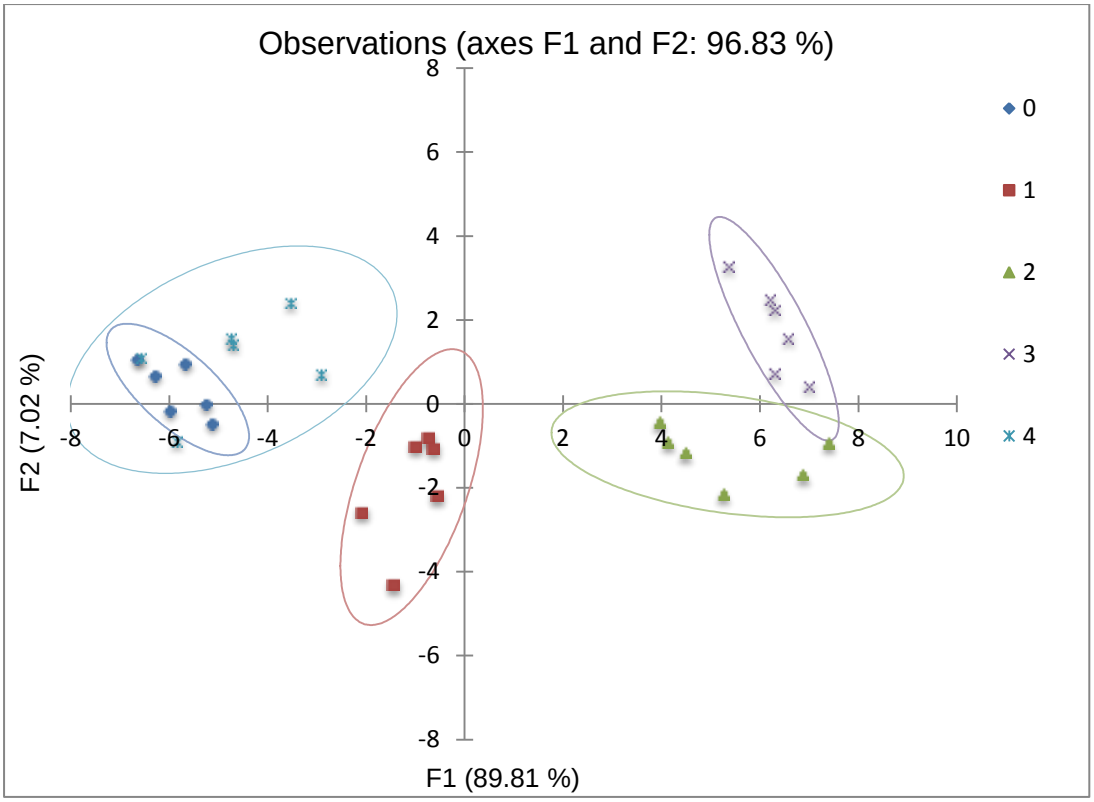

Fig. 6 Discriminant analysis (DA) observations chart of 'Grammy Smith' apples with different scald severity.

PAPER 4

Minimum Exposure Period for Dynamic Controlled Atmospheres to Control Superficial Scald in 'Granny Smith' Apples for Long Distance Supply Chains

Abstract

This study was conducted to investigate the potential of dynamic controlled atmospheres (DCA) to control superficial scald in pre-optimally and optimally harvested 'Granny Smith' apples over two growing seasons. The critical minimum period for DCA to control superficial scald was also investigated. Fruit was stored in DCA at 0 °C for 5 d up to 20 weeks followed by 6 or 10 weeks at -0.5 °C simulated handling conditions for long distant supply chains plus 7 d at 20 °C. The scald potential for each storage time in each season was assessed by storing fruit in RA and used as the control. For determining critical minimum period, the shipment of 10 weeks at regular atmosphere (RA) was used. Superficial scald incidence and quality parameters such as total soluble solids (TSS), titratable acidity (TA), fruit firmness and ground color were measured. The evolution of scald associated metabolites (α-farnesene, 6-methyl-5-hepten-2-one (MHO) and ethylene) was also monitored. The results showed that DCA controlled superficial scald in pre-optimally and optimally harvested fruit in both growing seasons. For all the storage regimes, DCA stored fruit appeared to have a significantly ($p<0.05$) higher ground colour and fruit firmness compared to RA stored fruit. All the fruit stored at DCA had significantly lower concentrations of ethylene, α-farnesene and MHO. The Pearson correlation showed a strong relationship among scald associated metabolites. Correlation coefficient for α-farnesene and MHO of fruit stored in RA and DCA were 0.831 and 0.822, respectively, while those for scald incidence and MHO were 0.863 and 0.365 in RA and DCA, respectively. Our results suggest that DCA was effective in controlling superficial scald development in both pre-optimally and optimally harvested 'Granny Smith' apples when shipping period under RA was limited to 6 weeks after DCA storage . However, storing fruit for 10 weeks at RA after DCA treatment increased the risk of superficial scald development.

Keywords: *Malus domestica*, dynamic controlled atmosphere, superficial scald, maturity, α-farnesene, ethylene, 6-methyl-5-hepten-2-one

1. Introduction

Superficial scald is a major postharvest physiological disorder reducing the quality of 'Granny Smith' apples (Malus x domestic Borkh.). Susceptibility of fruit to superficial scald varies among cultivars. Cultivars such as 'White Angel', 'Idared', 'Gala', 'Émpire' and 'Golden Delicious' are scald resistant whilst superficial scald is more prevalent on 'Rome Beauty', 'Law Rome', 'Cortland', 'McIntosh' and 'Granny Smith' (Fernández-Trujillo et al., 2003; Rao et al, 1998). Despite this knowledge, biotechnology research has thus far been unsuccessful in developing scald resistant 'Granny Smith' apple which has high market value due to its desirable organoleptic properties. Consequently, the industry heavily relies on chemical treatments for controlling scald.

Fruit maturity plays an integral role in postharvest storage potential, flavour development and scald susceptibility (Echeverria et al., 2004). Generally, scald susceptibility declines with advancing maturity (Lurie and Watkins, 2012; Wang and Dilley, 1999). Previous experimental research has demonstrated that scald severity has inverse relationship with maturity (Meir and Bramlage, 1988). However, it is not always possible to harvest at optimal maturity due to labour shortage and poor fruit quality associated with delayed harvest. As a result, apples are normally harvested at both pre-optimal and optimal maturities. To maintain quality and control scald, chemical treatments such as diphenylamine (DPA) and 1-methylcyclopropene (1-MCP) are used (Hall et al., 1961; Fan et al., 1999). However, the use of chemical treatments at postharvest is becoming unpopular. In fact, high paying markets reject chemical treatments on food due to health concerns (Pechous & Whitaker, 2004). Controlled atmospheres (CA) have been used; however, scald is not completely controlled in CA (DeLong et al., 2007).

An improved version of CA termed dynamic controlled atmosphere (DCA) has been identified as a potential method (DeLong et al., 2007; Zanella et al., 2005). Several researchers have used DCA on different apple cultivars such as 'Gala', 'Golden Delicious', 'Pinova', 'Idared' (Mattheis et al., 1998; Zanella et al., 2008; Gabioud et al., 2009). However, no DCA protocol is available for 'Granny Smith' apples particularly for export orientated fruit industries such as South Africa which have distant markets requiring shipment period of 6 weeks at RA. Currently, there is no information on whether fruit stored in DCA before shipment can be scald free. Moreover, the minimum period that fruit should be stored at DCA for extended shipment period of 10 weeks remains unknown. The objective of this study was

to assess the potential of DCA to control superficial scald in pre-optimally and optimally harvested 'Granny Smith' apples. The critical minimum period for DCA to control scald was also investigated.

2. Materials and methods

2.1 Fruit source and treatments

The study was performed on pre-optimally and optimally harvested 'Granny Smith' apples during 2013 and 2014 growing seasons. Fruit free from visible external damage and blemishes were hand-picked from Valley Green Farm in Grabouw (34^0 12'12" S, 19^0 02'35" E), South Africa, at 165 and 172 days after full bloom (DAFB) (which are commonly considered in the fruit industry as pre-optimal (starch breakdown = 12.5%; firmness = 82 N) and optimal maturity (starch breakdown = 36.3%; firmness = 79 N), respectively. Uniformly sized fruit with diameter of 70±2 mm and mass of 160±5 g were randomly divided into 3 replications of 100 fruit each. The chlorophyll fluorescence non-destructive monitoring system (HarvestWatch, Satlantic Inc, Halifax, Canada) with an ability to predict and indicate low oxygen limit (LOL) was used to determine DCA set points (Prange et al., 2003; Wright et al., 2012). In this study, the DCA was established within 48 h after harvest, using compressed air and CO_2 plus N_2 from a membrane generator (Isosep, Isolcell, Italy). Accordingly, the gas composition of the storage chamber was analysed at 90 min intervals and adjusted when necessary. Generally, the O_2 levels ranged between 0.3% to 0.5% whilst CO_2 was maintained at 1% and 95% RH. The lowest O_2 set point was determined by identifying the O_2 partial pressure where an inflection in the fluorescence signal was detected, and then by increasing O_2 by 0.3% as a safety factor (Weber et al., 2015). Chlorophyll fluorescence was monitored and automatically adjusted following fluorescence signal as reported by Prange et al. (2007). Once the fluorescence signal is detected, almost 0.3% of O2 was added to the atmosphere until fluorescence signal is reduced to its initial value (Prange et al., 2007). To simulate possible South African industry scenarios, DCA storage regimes ranged between 5 d to 20 w followed by a 6 w storage period at RA (-0.5 °C, 95% RH) to simulate shipment period. RA storage was used as a control treatment.

To determine the minimum exposure period for dynamic controlled atmospheres to control superficial scald during a 10 w shipment period, another set of 3 replications of 100

fruit was used. Fruit was stored in DCA from 5 d up to 20 w followed by 10 w shipment period.

2.2 Assessment of fruit quality

Fruit quality was assessed at 7 d after storage at ambient conditions (20 °C and 65% RH). In the 10 w trial, scald incidence was the only assessment conducted whilst all other quality, physiological and biochemical assessments were conducted on the 6 w-trial. Scald incidence was recorded as the percentage of fruit with superficial scald symptoms. Ten fruit from each replication were used for quality assessment. Texture Analyser (Tensilon model UTM-4L, Tokyo Measuring Instruments Co., Ltd., Japan) with a 11.1 mm compression probe was used to measure fruit firmness. Operating conditions of the instrument were: pre-test speed 1.5 mm s^{-1}, 0.5 mm s^{-1} test speed, 10.0 mm s^{-1} post-test speed, and 0.20 N trigger force. Two measurements on opposite sides of each fruit aligned horizontally from the stem end to the apex were taken. Fruit firmness (N) was taken as force of compression. Ground color was assessed using Minolta Chroma Meter CR-300 (Minolta Corp, Osaka, Japan). Hue angle [°H= arctan (b*/a*)] was calculated and used to measure ground colour (Pathare et al., 2013). Total soluble solids (TSS) and titratible acidity (TA) were measured after fruit was juiced using a LiquaFresh juice extractor (Mellerware, South Africa). TA was determined using a Metrohm 862 compact titrosampler (Herisau, Switzerland), and the results were expressed as milligram per litre of malic acid. TSS (ºBrix) was measured using a digital refractometer (Atago, Tokyo, Japan). According to Visser et al. (1968), the poor correlation between sweet taste and TSS can be ascribed to the over- or underestimation of sweet taste in the presence of a low or high sour taste, respectively. TSS/TA ratio, was therefore used as a reliable indicator of fruit maturity and sweetness (Magwaza and Opara, 2015). Moreover, TSS/TA is closely linked to consumer organoleptic perception of sweet and sour fruit compared to only TSS or TA separately (Hamadziripi et al., 2014; Magwaza and Opara, 2014).

2.3 Ethylene production and headspace volatile analysis

Fruit ethylene production was measured as described by Öz and Ergun (2009) with slight modifications. Individual fruit was weighed using an electronic balance (Mettler Toledo, Switzerland, 0.01g accuracy), and thereafter enclosed in 1 L airtight glass jar with a lid containing a rubber septum. After 1 h incubation at 20 °C, ethylene concentration in the

glass jars was measured using an infrared ethylene analyser (ICA56 ppm, United Kingdom). Ethylene was thereafter calculated and expressed as mean ± SE ($\mu L C_2H_4 kg^{-1}h^{-1}$).

For volatile analysis, fresh peel (5 g) was weighed into a 20 mL solid phase microextraction (SMPE) glass vials. 10 µL of 3-octanol internal standard was added, and the vials were sealed. Vial headspace was analysed according to Mayuoni-Kirshinbaum et al. (2012) and Caleb et al. (2013). The vials were equilibrated for 10 min at 50 °C in a CTC autosampler incubator (Leap Technologies, Carboro, NC). After equilibration, a 50/30 µm divinylbenzene/-carboxen/-polydimethylsiloxane coated fibre was then exposed to the sample headspace for 20 min at 50 °C. After extraction, the trapped volatile compounds from the fibre coating were desorbed for 2 min in the injection port of the gas chromatograph operated in a splitless mode. The temperature was maintained at 250ºC for the injection. The fibre was cleaned after each sample heating for 10 min in the fibre conditioned station maintained at 270ºC. Chromatographic separation of the extracted volatiles was performed on a Agilent 6890N (Agilent, Palo Alto, CA) connected through a transfer line to a Agilent 5975B MS (Agilent, Palo Alto, CA) mass spectrometer detector. The GC–MS system was equipped with a polar DB-FFAP column from J&W (part number 122-3263) with the following dimensions: (60 m length; 250 µm internal diameter; and 0.5 µm film thickness). Helium was used as carrier gas at a flow rate of 1.3 mL min^{-1}. The oven temperature program was as follows: initial temperature of 40 °C for 5 min; then ramped at 5 °C min^{-1} up to a final temperature of 230 °C with a final hold time of 6 min. The ion source and quadropole were maintained at 240 °C and 150 °C, respectively. The transfer line temperature was maintained at 280 °C. Alpha-farnesene and MHO were identified by a library search and quantified using abundance characteristic ion 93 and 108, respectively. Generally, α-farnesene gave a single peak at 22.2 min while MHO gave a peak at 16.6 min. A reading of 10^4 in abundance was defined as one unit and expressed as U g^{-1} (Ju and Curry, 2000).

3. Statistical analysis

The results were presented as mean (±S.E) values. Analysis of variance (ANOVA) was carried out using STATISTICA 12 (StatSoft, Inc. Oklahoma, USA) according to Duncan's multiple range test. Graphical data presentations were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc. San Diego, USA).

4. Results and discussion

4.1 Superficial scald incidence

Scald development in 'Granny Smith' apples was recorded as percentage of scalded fruit (Table 1; Fig. 1). In this study, an interaction among harvest maturity, growing season, storage time and storage condition (A*B*C*D) had a significant ($p<0.0001$) effect on superficial scald development (Table 1, Fig 1) This interaction could be explained by the markedly higher scald incidence in RA stored fruit as opposed to lower scald incidence in DCA stored fruit (Table 1, Fig 1). In the trial where the 6 weeks RA shipping was applied after DCA, the two growing seasons, scald developed on control fruit (RA) after 4 w whilst DCA prevented scald development for the whole storage duration for both maturities. Moreover, fruit harvested at optimal maturity appeared to be less prone to scald development compared to those harvested at pre-optimal maturity. The observed results are consistent with Zanella et al. (2005) who reported a complete scald control in 'Granny Smith' apples after DCA storage of 6 month and 14 d shelf-life. The efficacy of DCA to control superficial scald even during shipment period is an added advantage for South African Fruit Producers. Unlike internal browning and breakdown that has been reported in DCA stored 'Abbè Fetél' pears (Vanoli et al., 2010), 'Granny Smith' apples had a better quality.

When the 10 w RA shipping simulation period was applied after DCA, the first season revealed that the pre-optimally harvested fruit should be stored for a minimum of 8 w whilst optimally harvested fruit should be stored for at least 20 w at DCA before RA storage of 10 w (Fig. 1A). However, for the second season, even though scald incidence declined with an increase in fruit exposure to DCA, scald was never completely controlled (Fig. 1B). This finding indicates the seasonal influence on the treatment. These results are consistent with Hernandez and Torres (2014) who reported almost 100% scald incidence in 'Granny Smith' apples stored at DCA before shipment period of 12 w at RA storage. Based on this result, it could be argued that DCA storage does not prevent superficial scald development, but rather delays the symptoms and depends on the RA period post the DCA treatment.

4.2 TSS, TA and TSS/TA

Significant differences ($p<0.05$) were found in TSS content (expressed as °Brix) between the investigated storage conditions when evaluated after the 6 week RA period and

ripening. TSS content was significantly (p=0.0015) influenced by the interaction among harvest maturity, growing season, storage time and storage condition (A*B*C*D) (Table 1). The results suggest that TSS content is higher in fruit stored at RA compared to DCA stored fruit for both maturities, moreover, TSS increased with storage time. This finding is contrary to higher TSS content previously reported in low oxygen storage. For instance, DeLong et al. (2007) found a higher TSS content in 'Cortland' apples stored in DCA compared to those stored in standard CA. Tran et al. (2015) also reported a higher TSS content on 'Greenstar' apples stored in DCA compared to CA storage for 10 months and subsequent 7 d shelf-life. It is possible that starch breakdown in DCA treated fruit was more inhibited that in RA stored fruit and hence a slower conversion to simple sugars contributing to the TSS. Should the shelf-life have been longer perhaps the TSS value would have increased further.

Although the interaction between harvest maturity, growing season, storage time and storage condition (A*B*C*D; p=0.0319) had a significant influence on titratable acidity content (TA, expressed as malic acid mg/100ml) after the 6 weeks RA shipping period and ripening, there was a notable decline during the investigated storage time and conditions (Table 1). The decline was more pronounced in RA stored fruit. This finding is in agreement with the reports of DeLong et al. (2007) and Gabioud et al. (2009) in which DCA better minimised loss of TA in 'Cortland' and 'Golden Delicious' apples during cold storage. DCA treatment resulted in lower TSS/TA ratio for both seasons and maturities, with a significant interaction (A*B*C*D; p=0.0007) amongst all the main factors. The retention of TA content and consequently reduced TSS/TA may be as a result of lower respiration rate in DCA stored fruit. In fact, the reduced TA content in scalded or RA stored fruit could be linked to cellular activity in which organic acids serve as substrates that enter Krebs cycle to gain energy for repairing the aging cell and membranes (Taiz and Zeiger, 2002).

4.3 Fruit firmness and ground colour

Fruit firmness is one of the important quality parameters in apple fruit. The loss of firmness in apples is the most noticeable change occurring during prolonged storage and it is closely linked to reduced water content and metabolic changes (García et al., 1998). Our results showed a combined significant effect of harvest maturity, storage condition and storage time (A*C*D; p=0.0046) together with growing season, storage condition and storage time (B*C*D; p=0.0112) on fruit firmness (Table 2 and 3) for the 6 week shipment and ripening data. Firmness loss seemed to be more pronounced in fruit harvested at optimal

maturity compared to those harvested at pre-optimal maturity (Table 2). In both 2013 and 2014 season, DCA maintained higher fruit firmness compared to RA (Table 3) even though it declined during storage in both RA and DCA storage conditions. This result is in agreement with the findings of Weber et al. (2015) who reported higher flesh firmness on 'Royal Gala' apples stored in DCA compared to those stored in CA. Based on these results, it is clear that reduced oxygen in DCA could indeed result to a delay in ripening process and consequently improve fruit quality.

Fruit background colour change from green to yellow, as measured by *hue angle*, is an important quality attribute that determines the market value of 'Granny Smith' apples. Generally, postharvest treatments and storage conditions should retard both chlorophyll degradation and biosynthesis of carotenoids (Kays, 1991). In this study, harvest maturity had a significant ($p=0.0058$) effect on fruit colour. Fruit harvested at pre-optimal maturity were greener compared to those harvested at optimal maturity (Fig. 2A) when measured after the DCA treatments and 6 w of RA plus 7 days shelf-life. Growing season and storage condition had a combined significant ($p=0.0078$) effect on fruit colour (Fig. 2B). This was probably influenced by high scald incidence in 2013 season and higher *hue angle* in DCA stored fruit. This is attributed to the fact that scald development leads to the loss of *hue angle* during storage, and the opposite is also true. A combined effect of storage time and storage condition (C*D; $p<0.0001$) and storage time and growing season significantly influenced hue angle (B*C; $p<0.0001$). *Hue angle* declined during storage, however, the reduction was minimal in DCA stored fruit when stored for longer than 2 weeks compared to those stored in RA (Fig. 3A). This finding suggests that DCA storage delays the ripening process and chlorophyll degradation (Tran et al., 2015). Similar results have been observed in 'Granny Smith' apples (Zanella, 2003) and 'd'Anjou' pears (Mattheis and Rudell, 2011) stored in low oxygen atmospheres. The ability of DCA to retain fruit colour is a highly desired quality attribute especially after shipment and at shelf-life.

4.4 Ethylene production

Ethylene production during storage has an influence on superficial scald associated physiological and biochemical changes, fruit quality and shelf-life (Ingle, 2001; Ju and Curry, 2002). In studies on cell wall degradation, Wei et al. (2010) and Ortiz (2011) reported that fruit firmness loss is caused by ethylene activated enzymes such as β-galactosidase, β-xylosidase, pectin endoglucanase and polygalacturose. Previous studies have also shown that

ethylene inhibiting postharvest treatments such as 1-MCP improve firmness and overall fruit quality (Mir et al., 1999; Cocci et al., 2014). In this study, storage condition and storage time had a significant interaction ($p<0.0001$) whilst harvest maturity and storage time also showed a significant interaction ($p=0.0363$) on ethylene production after 10 weeks shipping in RA and shelf-life. Ethylene production increased with storage time in both RA and DCA, however, it declined after 12 w in RA stored fruit (Fig. 4A). Moreover, ethylene production was higher in RA stored fruit compared to those stored in DCA regardless of both being stored in RA during shipment. At harvest, ethylene content did not differ between the maturities (Fig. 4B). This difference in ethylene production between RA and DCA stored fruit disappeared after 8 weeks of DCA storage and shipping plus ripening. Since ethylene is linked to ripening and senescence, it means that postharvest treatment indeed plays a vital role in controlling ethylene production. These results are comparable to Weber et al. (2015) who reported a reduced ethylene production rate in 'Royal Gala' apples stored in DCA for 8 months. The low oxygen levels in DCA reduce oxidative metabolism (Imahori et al., 2013) resulting to decreased 1-Aminocyclopropane-1-Carboxylate (ACC) oxidase activity and subsequently ethylene production (Brackmann et al., 2013; Weber et al., 2015). Reduced scald incidence in 'Granny Smith' apples stored in flow-through 1.5% O_2 CA was strongly associated with low ethylene production during storage (Whitaker et al. 1997). Similarly, the superior fruit quality in DCA stored 'Granny Smith' apples as found in this study could be linked to low ethylene production, a key element in postharvest performance of pome fruits.

4.5 Alpha-farnesene and MHO

Alpha-farnesene accumulation is closely associated with scald development in apples and pears (Lurie and Watkins, 2012). In this study, α-farnesene accumulation was highly influenced by both fruit maturity ($p<0.0001$) and storage time*storage condition (C*D; $p<0.0001$) after the 6 weeks shipping period and ripening (Fig. 5A and B). The high α-farnesene concentration in fruit harvested at pre-optimal maturity (Fig. 5A) was consistent with prevalence of scald in this fruit as opposed to lower concentration and reduced scald incidence in fruit harvested at optimal maturity (Lurie and Watkins, 2012; Wang and Dilley, 1999). Isidoro and Almeida (2006) also reported that higher α-farnesene accumulation in 'Rocha' pears harvested at pre-optimal compared to those harvested at optimal maturity. However, contrary findings have been reported for some apple cultivars. For instance, Barden and Bramlage (1994) reported a low α-farnesene concentration in pre-optimally harvested

'Cortland' apples. The difference in morphological and biochemical properties of cultivars could be the reason for contradictory findings.

The interaction between storage time and storage condition had a significant influence α-farnesene accumulation. Alpha-farnesene concentrations increased with storage time to maximum levels at 12 w in RA stored fruit (Fig. 5B). This trend possibly indicates that α-farnesene measurement during the 3rd to 12th week of storage would serve as an indicator for scald susceptibility in 'Granny Smith' apples. Interestingly, ethylene production followed exactly the same trend for this interaction of the main effects therefore this trend may be directly linked to ethylene production. Notably, the accumulation of α-farnesene was more pronounced in RA stored fruit compared to those stored in DCA. The trend of α-farnesene accumulation in RA differed from that of fruit stored in DCA, a phenomenon that has previously been reported by Isidoro and Almeida (2006) in 'Rocha' pears stored in air or CA. Interestingly, exposing 'Granny Smith' apples to <0.5% O_2 for 10 d before cold storage of 24 w reduced α-farnesene concentration (Sabban-Amin et al., 2011). Moreover, Whitaker (2000) reported a delayed α-farnesene accumulation in apples stored at low oxygen CA.

MHO results from α-farnesene oxidation and is reported to be directly involved in primary physiological and biochemical events leading to superficial scald development (Mir et al., 1999; Wang and Dilley, 2000). High MHO accumulation after the removal of fruit from cold storage coincides with scald symptoms in 'Cortland' (Mir et al., 1999) and 'Granny Smith' apples (Wang and Dilley, 2000). In this study, fruit maturity ($p=0.0053$) significantly affected MHO accumulation after the 6 weeks shipping period and ripening. Similar to α-farnesene accumulation, fruit harvested at pre-optimal maturity had higher MHO concentrations compared to those harvested at optimal maturity (Fig. 6A). This result corroborates with previous findings correlating α-farnesene and MHO accumulation (Wang and Dilley, 2000). Additionally, the higher α-farnesene and MHO concentrations in fruit harvested at pre-optimal maturity, as opposed to lower concentrations in optimal harvested fruit, validates the hypothesis of the involvement of these volatile compounds in scald etiology.

The interaction between storage time and storage condition also showed a significant effect on MHO accumulation. Analogous to α-farnesene accumulation, MHO increased with storage time in both storage conditions (Fig. 6B). However, it declined after 12 w in RA stored fruit whilst there was no significant difference in DCA stored fruit from 12 w to 20 w.

The pattern of MHO accumulation was the same as that of α-farnesene and ethylene production. MHO was markedly reduced by DCA compared to RA treatment (Fig. 6B). Our results could be comparable to those of Sabban-Amin et al. (2011) who found low MHO concentration on 'Granny Smith' apples pre-exposed to low oxygen levels before RA storage. To the best of our knowledge, this is the first research work reporting the influence of DCA on α-farnesene and MHO concentration. DeLong et al. (2007) and more recently, Tran et al. (2015) and Weber et al. (2015) showed that superficial scald is effectively reduced by DCA, but they did not report the evolution of scald associated volatile compounds such as α-farnesene and MHO during DCA storage. These findings will be highly instrumental in improving and optimising DCA technology for the storage of apples.

4.6 Correlation

Previous studies have shown that the inhibition of scald is strongly correlated to reduced ethylene synthesis and α-farnesene production (Watkins et al., 2000; Lurie et al., 2005). The correlations among α-farnesene, ethylene, MHO and superficial scald incidence were analysed for both RA and DCA storage. For RA, the Pearson correlation coefficient for α-farnesene and ethylene was 0.780, and 0.831 for α-farnesene and MHO concentration. For DCA, the correlation for α-farnesene and ethylene was 0.713, and 0.822 for α-farnesene and MHO concentration. The patterns of change between α-farnesene and MHO, and ethylene were similar to both RA and DCA stored fruit as reflected by strong and highly significant correlation coefficients. These results confirm the notion that α-farnesene synthesis is ethylene mediated (Watkins et al., 1995; Whitaker et al., 2000; Lurie et al., 2005). Moreover, the strong and highly significant correlation between α-farnesene and MHO is consistent with previous findings and thereafter confirming their biochemical relationship (Isidoro and Almeida, 2006; Pesis et al., 2010). Correlation coefficients for scald development and MHO were r= 0.863 and r= 0.365 in RA and DCA stored fruit, respectively. This result is similar to Wang and Dilley (2000) whose findings were consistent with the α-farnesene hypothesis that is based on a relationship between scald development and MHO concentration. However, for DCA stored fruit, the correlation between scald development and MHO was insignificant and very weak, this is probably due to MHO accumulation in DCA stored fruit reaching levels that are inconsistent with zero scald incidence in this fruit, and factors other than MHO may be involved in scald inhibition in addition to MHO.

5. Conclusion

This study has demonstrated that DCA is highly effective in maintaining the quality and controlling superficial scald in 'Granny Smith' apples harvested at pre-optimal and optimal maturities even when fruit shipping period of 6 weeks at RA is followed. However, storing apples for 10 weeks at RA after DCA treatment increases the risk of superficial scald development. The effectiveness of DCA to control superficial scald appears to be closely related to low ethylene, α-farnesene and MHO concentration.

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Table 1: Scald incidence, total soluble solids (TSS), titratable acidity (TA) and TSS/TA ratio of RA and DCA stored 'Granny Smith' apples harvested at pre and optimal maturity over two seasons. Fruit was stored at 0 °C for 0 d to 20 w followed by 6 w shipment period and 7 d shelf-life.

Season	Maturity	Storage Condition	Storage Time	%Scald	TSS	TA	TSS/TA
Season 1	Pre-optimal	RA	0D	0.00j	10.52z	1.46def	07.25y
			5D	0.00j	11.95u-y	1.12m-t	10.72m-r
			2W	0.00j	12.10u-x	1.07p-v	11.34h-o
			4W	85.67e	11.55u-z	1.05op-x	11.02k-p
			8W	94.18bc	11.60u-y	1.03p-x	11.32h-o
			12W	98.33a	12.15u-w	0.90x	13.64cde
			16W	99.40a	12.25un	0.84y	14.68bc
			20W	100.00a	12.40un	0.75yz	13.88cd
		DCA	0D	0.00j	10.52z	1.46 def	07.25y
			5D	0.00j	11.73y	1.14 k-r	10.31nt
			2W	0.00j	11.95x	0.96 t-z	12.48h-i
			4W	0.00j	11.47t-y	1.12 m-t	10.26ot
			8W	0.00j	11.97s-y	1.11 m-t	10.77m-q
			12W	0.00j	11.97s-y	1.20 hp	10.15otu
			16W	0.00j	11.77s-v	1.12 m-t	10.81m-q
			20W	0.00j	11.87u-z	1.14 jpq	10.45m-s
	Optimal	RA	0D	0.00j	10.83z	1.25h-l	08.68w
			5D	0.00j	12.95c-f	1.21h-o	10.66m-q
			2W	0.00j	12.90d-g	1.31f-j	09.94p-v
			4W	46.42g	12.40m-r	1.06p-w	11.67gm
			8W	92.40c	12.65e-n	1.12m-t	11.25h-o
			12W	97.63a	12.30l-t	1.34e-i	09.18tw
			16W	100.00a	12.80d-i	1.42d-g	08.98u-w
			20W	100.00a	12.30m-t	0.93v-z	13.17fd
		DCA	0D	0.00j	10.83z	1.25h-l	08.68w
			5D	0.00j	13.30abc	1.30h-k	10.24ot
			2W	0.00j	13.56a	1.08m-v	12.51e-h
			4W	0.00j	12.76d-j	1.13l-s	11.34h-o
			8W	0.00j	13.46ab	1.16pq	11.59hm
			12W	0.00j	13.18cd	1.43de	09.26vw
			16W	0.00j	12.96c-f	1.65bc	07.62xy
			20W	0.00j	12.96c-f	1.16k-r	11.12j-p
Season 2	Pre-optimal	RA	0D	0.00j	11.10z	1.54cd	07.21y
			5D	0.00j	13.03cde	1.42d-g	09.21tw
			2W	0.00j	12.70d-l	1.14k-r	11.11j-p
			4W	30.11i	12.76f-l	1.03px	12.39f-i
			8W	88.38d	12.46h-q	1.74b	07.13y
			12W	97.87a	12.47h-q	1.75b	07.13y
			16W	100.00a	12.63e-n	1.22h-n	10.46m-s
			20W	100.00a	12.36m-s	0.78z	15.77a
		DCA	0D	0.00j	11.10z	1.54cd	07.21y
			5D	0.00j	13.10cd	1.48de	08.84vw
			2W	0.00j	12.83d-h	1.10m-t	11.64gm
			4W	0.00j	13.10bd	1.07n-v	12.32f-j
			8W	0.00j	12.73d-j	1.78a	05.77z
			12W	0.00j	12.60mn	1.68a	05.72z
			16W	0.00j	12.67em	1.35eh	09.45rtw
			20W	0.00j	12.57f-o	1.04rxy	12.09f-l

Table 1 Cont'

	Optimal	RA	0D	0.00j	12.16ouv	1.29h-l	9.41stw
			5D	0.00j	12.76d-j	0.93v-z	13.73cd
			2W	0.00j	12.83d-h	1.11m-u	11.57mn
			4W	41.30h	11.76w	1.31f-j	9.59qtw
			8W	71.27f	12.43h-q	0.96 s-z	12.89df
			12W	94.98b	12.10u-x	0.94 u-z	12.83dfg
			16W	99.20a	11.43m-s	0.83y	13.79cd
			20W	100.00a	12.26o-w	0.81z	15.13ab
		DCA	0D	0.00j	12.16o-w	1.29h-l	09.41st
			5D	0.00j	12.53m-p	0.90wx	13.93cd
			2W	0.00j	12.60mn	1.11m-t	11.28h-o
			4W	0.00j	12.76d-j	1.31h-j	10.32nt
			8W	0.00j	12.33m-t	1.00qx	12.30f-j
			12W	0.00j	12.30m-t	1.18ip	10.47n-s
			16W	0.00j	11.93t-y	1.10m-v	10.86l-p
			20W	0.00j	12.00r-x	0.98px	12.21f-l

	Pr>F			
Maturity (A)	<0.0001	<0.0001	<0.0001	<0.0001
Season (B)	<0.0001	<0.0001	<0.0001	0.6297
Storage Time (C)	<0.0001	<0.0001	<0.0001	<0.0001
Storage Condition (D)	<0.0001	<0.0001	<0.0001	<0.0001
A*B	<0.0001	<0.0001	<0.0001	<0.0001
A*C	<0.0001	<0.0001	<0.0001	<0.0001
B*C	<0.0001	<0.0001	<0.0001	<0.0001
A*D	<0.0001	0.0118	0.0014	0.0026
B*D	<0.0001	0.0272	0.1599	0.3945
C*D	<0.0001	<0.0001	<0.0001	<0.0001
A*B*C	<0.0001	<0.0001	<0.0001	<0.0001
A*B*D	<0.0001	0.0017	0.4082	0.0004
A*C*D	<0.0001	<0.0001	0.0109	0.7595
B*C*D	<0.0001	<0.0001	0.0045	0.0091
A*B*C*D	**<0.0001**	**<0.0015**	**0.0319**	**0.0007**

Table 2: Effects of the interaction between harvest maturity, storage condition and storage time on firmness of 'Granny Smith' apples stored at 0 °C for 0 d to 20 w followed by 6 w shipment period and 7 d shelf-life.

Maturity	Storage Condition	Storage Time	Firmness (N)
Pre-optimal	RA	0D	81.99ab
		5D	77.01c-f
		2W	72.52ghi
		4W	66.57lk
		8W	64.45l
		12W	61.16m
		16W	57.60n
		20W	51.54o
	DCA	0D	81.99ab
		5D	79.45bd
		2W	77.53cde
		4W	76.27def
		8W	73.63f-i
		12W	72.10gj
		16W	70.88ji
		20W	70.45ji
Optimal	RA	0D	79.14a
		5D	74.50egh
		2W	71.70hj
		4W	68.82jk
		8W	66.18kl
		12W	55.65n
		16W	50.83o
		20W	44.70p
	DCA	0D	79.14a
		5D	80.25abc
		2W	77.78cde
		4W	76.73def
		8W	75.47eg
		12W	73.70f-i
		16W	71.85hj
		20W	71.02hj

	Pr>F
Maturity (A)	0.1222
Season (B)	0.5244
Storage Time (C)	<0.0001
Storage Condition (D)	<0.0001
A*B	0.4181
A*C	0.0039
B*C	0.0256
A*D	<0.0001
B*D	0.4521
C*D	<0.0001
A*B*C	0.0528
A*B*D	0.8730
A*C*D	**0.0046**
B*C*D	0.0112
A*B*C*D	0.4659

Table 3: Effects of the interaction between growing season, storage condition and storage time on firmness of 'Granny Smith' apples stored at 0 °C for 0 d to 20 w followed by 6 w shipment period and 7 d shelf-life.

Season	Storage Condition	Storage Time	Firmness (N)
2013	RA	0D	81.79ab
		5D	74.26e-f
		2W	72.24gi
		4W	66.14jk
		8W	64.08k
		12W	58.19l
		16W	56.16l
		20W	51.12m
	DCA	0D	81.79ab
		5D	80.06ac
		2W	78.31cd
		4W	77.17cde
		8W	75.27dfg
		12W	73.16fgh
		16W	71.74gi
		20W	70.77hi
2014	RA	0D	79.34a
		5D	77.25cde
		2W	71.98gi
		4W	69.25ij
		8W	66.55jk
		12W	58.62l
		16W	52.27m
		20W	45.11n
	DCA	0D	79.34a
		5D	79.63bc
		2W	77.00cde
		4W	75.82df
		8W	73.82e-h
		12W	72.64fi
		16W	70.98hi
		20W	70.69hi

	Pr>F
Maturity (A)	0.1222
Season (B)	0.5244
Storage Time (C)	<0.0001
Storage Condition (D)	<0.0001
A*B	0.4181
A*C	0.0039
B*C	0.0256
A*D	<0.0001
B*D	0.4521
C*D	<0.0001
A*B*C	0.0528
A*B*D	0.8730
A*C*D	0.0046
B*C*D	**0.0112**
A*B*C*D	0.4659

Table 4: This table shows the significance levels of the factors and their interactions of the figures presented below

Source	Significance level				
	Scald	**Hue Angle**	**Ethylene**	**A-farnesene**	**MHO**
Maturity (A)	<0.0001	**0.0058**	0.2587	**<0.0001**	**0.0053**
Season (B)	<0.0001	0.9623	0.3372	0.3136	0.0781
Storage Time (C)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Storage Condition (D)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
A*B	<0.0001	0.1291	0.8360	0.5453	0.0816
A*C	<0.0001	0.3124	**0.0363**	0.8872	0.3081
B*C	<0.0001	**<0.0001**	0.9994	0.8747	0.8272
A*D	<0.0001	0.6261	0.9589	0.1774	0.6574
B*D	<0.0001	**0.0078**	0.6197	0.0781	0.6001
C*D	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
A*B*C	<0.0001	0.3169	0.9986	0.9451	0.7956
A*B*D	<0.0001	0.7256	0.6536	0.3774	0.9844
A*C*D	<0.0001	0.7858	0.8886	0.9864	0.1283
B*C*D	<0.0001	0.1868	0.9994	0.8144	0.9978
A*B*C*D	**<0.0001**	0.6937	0.9932	0.6637	0.9301

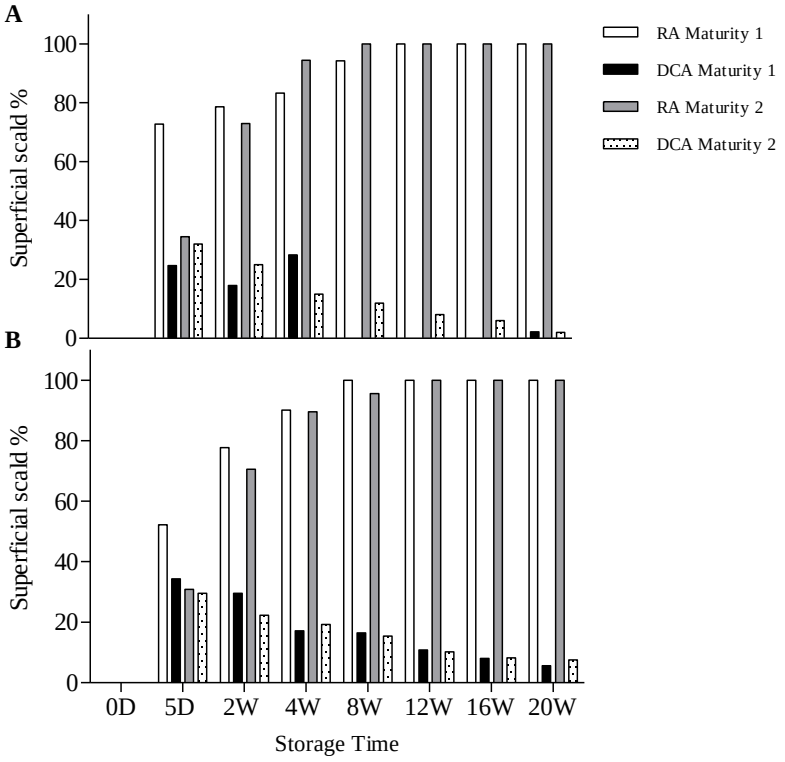

Fig. 1 The effect of the interaction between storage time, growing season, storage condition and harvest maturity on superficial scald incidence of 'Granny Smith' apples during season 1 (A) and season 2 (B). Fruit were stored at 0 °C for 0 d to 20 w followed by 10 w shipment period and 7 d shelf-life.

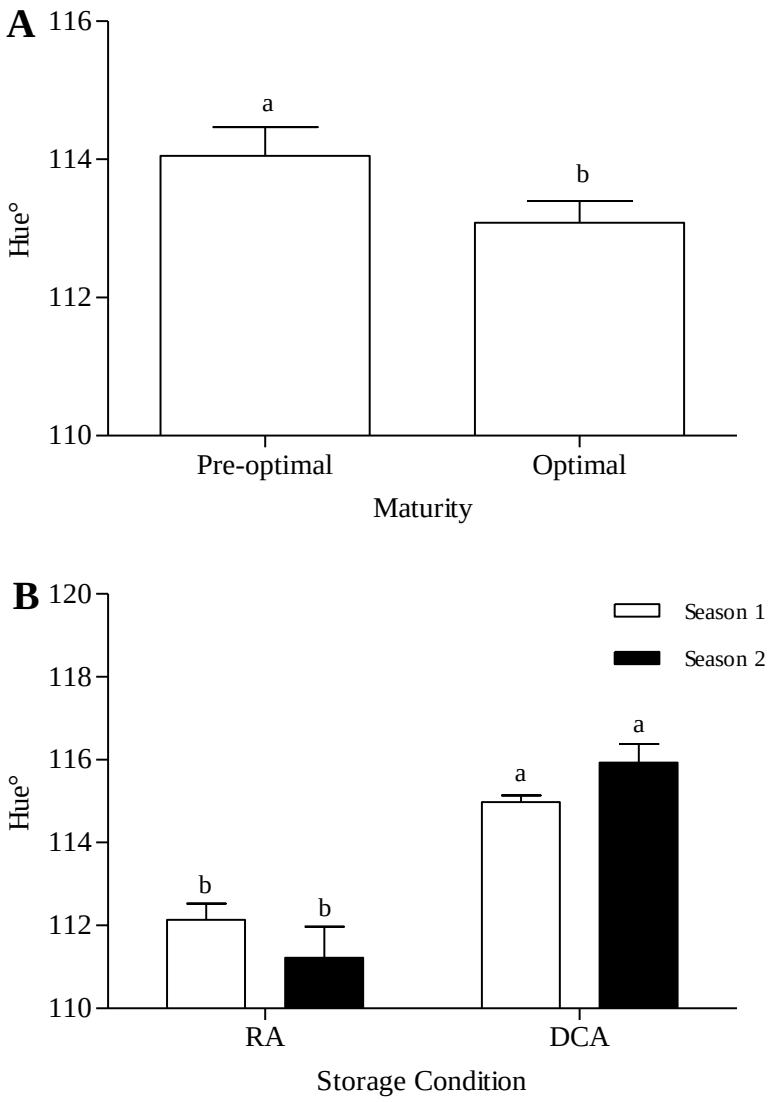

Fig. 2 Effects of harvest maturity (A) and the interaction between growing season and storage condition (B) on hue angle of 'Granny Smith' apples stored at 0 °C for 0 d to 20 w followed by 6 w shipment period and 7 d shelf-life.

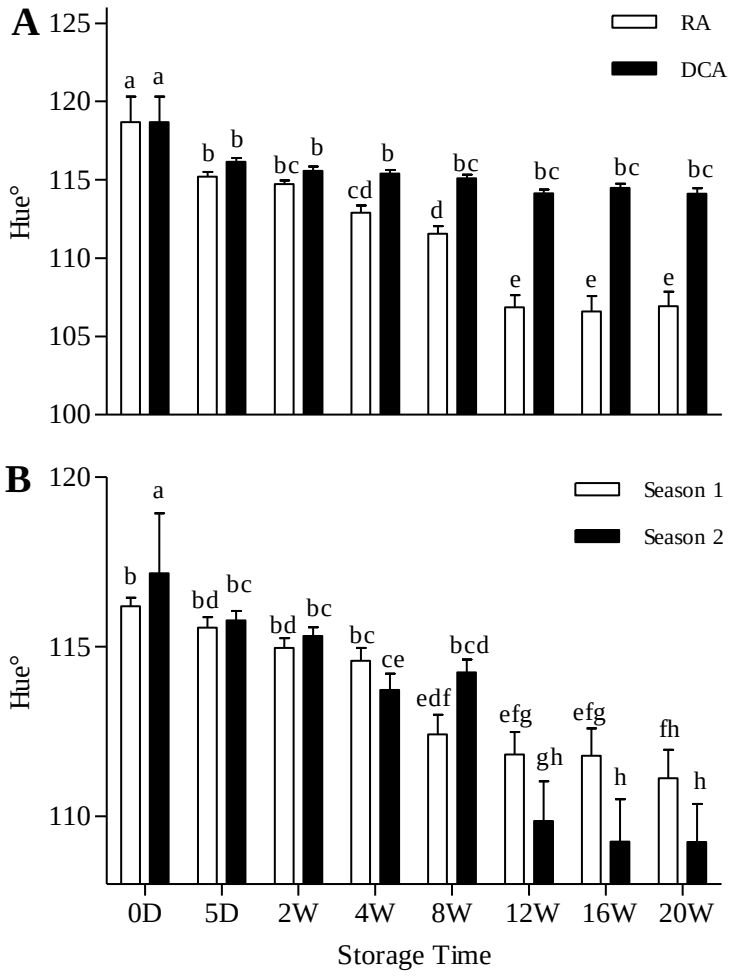

Fig. 3 Effects of the interaction between storage time and storage condition (A) and the interaction between storage time and growing season (B) on hue angle of 'Granny Smith' apples stored at 0 °C for 0 d to 20 w followed by 6 w shipment period and 7 d shelf-life.

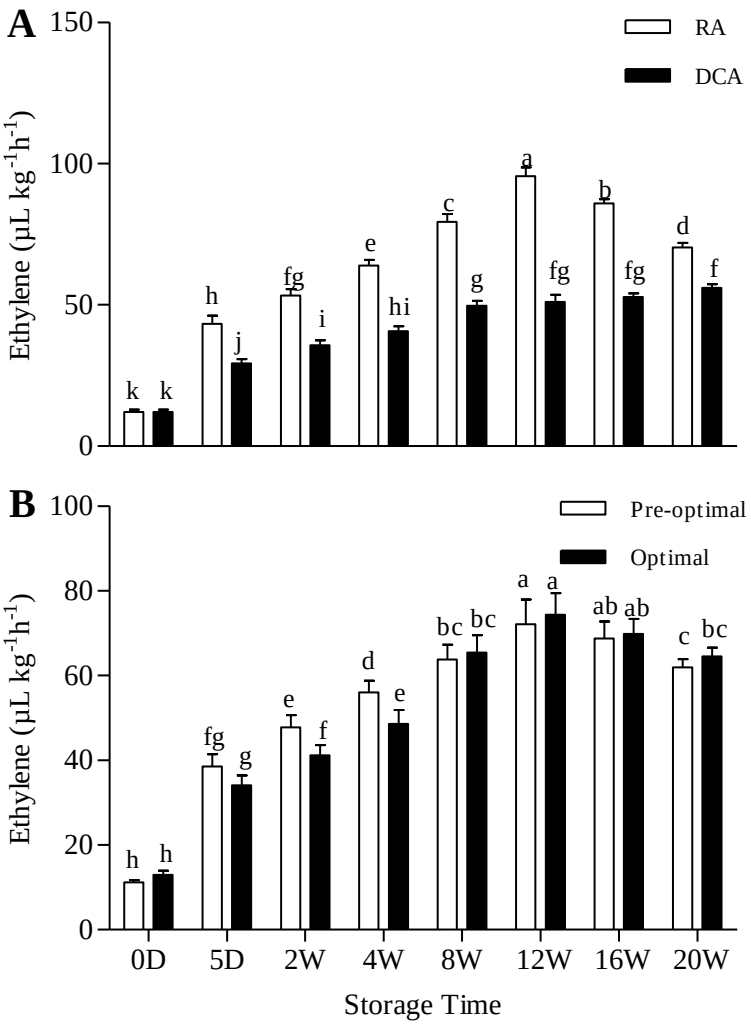

Fig. 4 Effects of the interaction between storage time and storage condition (A) and the interaction between storage time and harvest maturity (B) on ethylene production of 'Granny Smith' apples stored at 0 °C for 0 d to 20 w followed by 6 w shipment period and 7 d shelf-life.

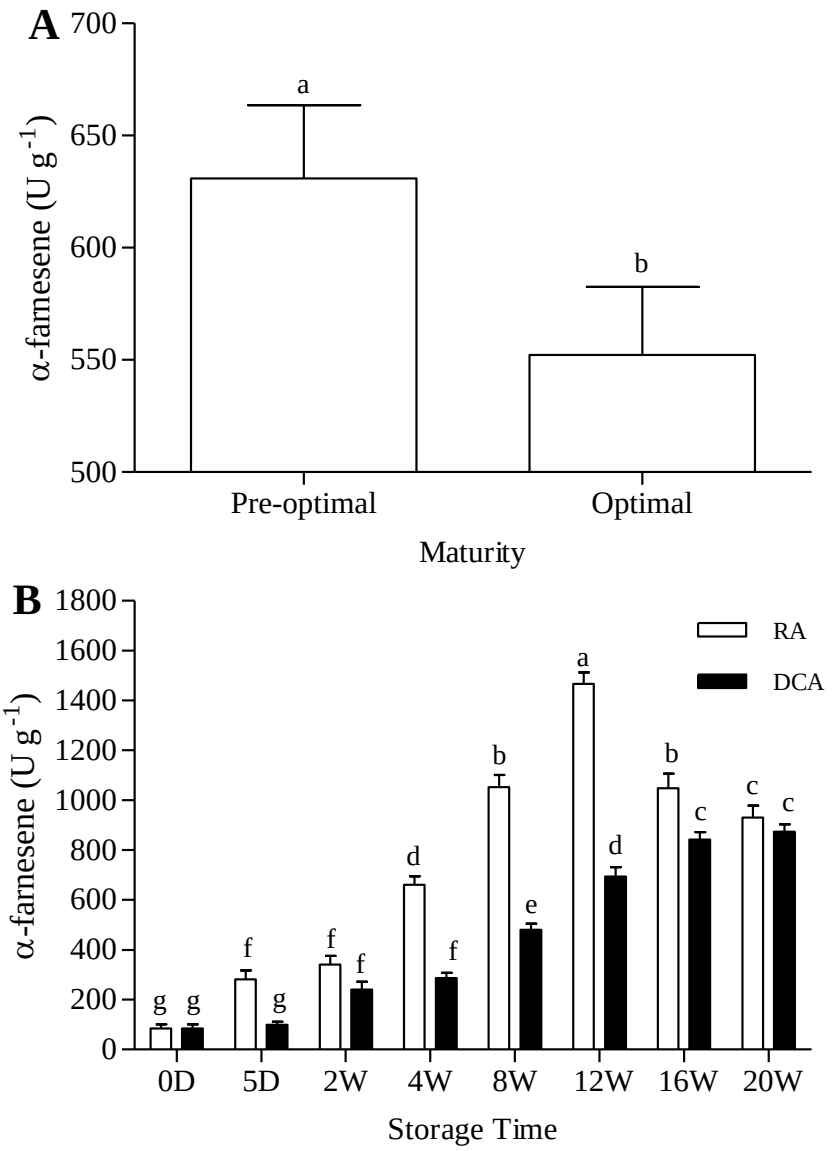

Fig. 5 Effects of the interaction between storage time and storage condition (A) and the interaction between storage time and harvest maturity (B) on ethylene production of 'Granny Smith' apples stored at 0 °C for 0 d to 20 w followed by 6 w shipment period and 7 d shelf-life.

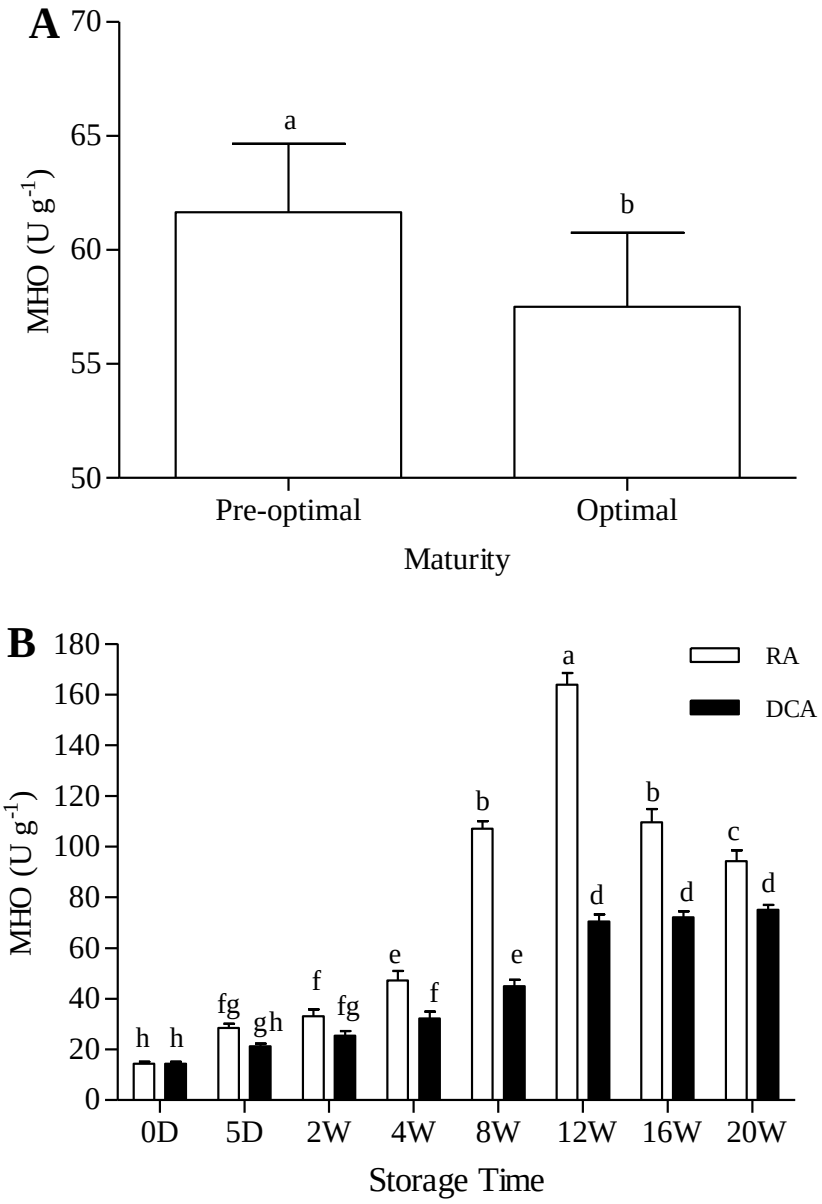

Fig. 6 Effects of harvest maturity (A) and the interaction between storage time and storage condition (B) on MHO production of 'Granny Smith' apples stored at 0 °C for 0 d to 20 w followed by 6 w shipment period and 7 d shelf-life.

PAPER 5

Impact of dynamic controlled atmospheres on reactive oxygen species, antioxidant capacity and phytochemical properties in 'Granny Smith' apples

Abstract

This study was conducted to investigate the effect of dynamic controlled atmospheres (DCA) on antioxidant capacity and reactive oxygen species (ROS) of 'Granny Smith' apples harvested at pre-optimal and optimal maturity over two growing seasons. Fruit were stored in DCA (<0.5% O_2; 1% CO_2) for up to 20 weeks at 0 °C and refrigerated air (RA) was used as a control treatment. Antioxidant capacity and lipid peroxidation were spectrophotometrically measured. ROS were measured by confocal laser-scanning microscopy on apple peel treated with fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate. Principal component analysis (PCA) and Pearson correlation were used to investigate major changes and relationship among the studied variables. Fruit stored in DCA were characterized by higher antioxidant capacity, ascorbic acid and total phenolics concentration. PCA displayed two clusters that could easily be identified as DCA and RA stored fruit. Positive correlation stores for lipid peroxidation and ROS corresponded with RA stored fruit whilst strong negative stores for antioxidant capacity, ascorbic acid and total phenolics corresponded with DCA stored fruit. Pearson correlation showed a strong relationship between ascorbic acid, phenolics and antioxidant capacity. Lipid peroxidation and ROS also showed a significant ($p<0.05$) positive correlation. This study showed that the high fruit quality in DCA correlates with higher antioxidant capacity, total phenolics and ascorbic acid concentrations in this fruit.

Keywords: *Malus domestica*, ascorbic acid, total phenolics, antioxidant capacity, reactive oxygen species, lipid peroxidation

1. Introduction

Dynamic controlled atmospheres (DCA) have been shown as the potential technique to control superficial scald incidence and improve postharvest quality attributes in pome fruits. DCA has an ability to maintain fruit firmness, acidity and overall fruit quality (DeLong et al., 2007; Gabioud et al., 2009; Tran et al., 2015; Weber et al., 2015). However, the biochemical mechanisms used by DCA in controlling superficial scald and improving

postharvest quality of apples is not yet understood. Superficial scald is presumed to be an oxidative stress; this is attributed to the reduced incidence after the application of antioxidants such as diphenylamine (DPA) or low oxygen storage (Lurie and Watkins, 2012). Moreover, α-farnesene oxidation products such as conjugated trienes (CTs) and 6-methyl-5-hepten-2 one (MHO) are cited as the main causal agents of superficial scald (Rowan et al., 1995; Mir et al., 1999; Wang and Dilley, 2000).

Apart from α-farnesene hypothesis, superficial scald susceptibility or resistance could also be influenced by the antioxidant pool of the fruit during storage (Anet, 1972). For instance, Barden and Bramlage (1994a) reported high antioxidant pool to be highly associated with low α-farnesene and consequently reduced scald incidence in apples. Meir and Bramlage (1988) also showed that the high storage potential of optimally harvested 'Cortland' apples is attributed to increasing antioxidant capacity in the cuticle during maturation. A strong relationship between scald symptoms and phenol concentration has also been reported in 'Delicious' apples (Ju et al., 1996). Moreover, a scald resistant 'Golden Delicious' is reportedly having a higher phenol concentration compared to scald susceptible 'Cortland' apples (Ju and Bramlage, 1999). A recent investigation has compared the reactive oxygen species (ROS) accumulation with activities of enzymes that scavenge toxic compounds on scald susceptible tissues of 'Granny Smith' apples (Sabban-Amin et al., 2011). Interestingly, low ROS concentration corresponded with a higher enzyme activity and lower scald incidence. Lipid peroxidation is another biochemical disorder that often precedes the expression of chilling injury or superficial scald symptoms (Lyons, 1973). In fact, lipid peroxidation increases with scald incidence and severity in 'Fuji' apples (Lu et al., 2014). Rao et al. (1998) also found a relationship between lipid peroxidation and scald incidence in 'White Angel' x 'Rome Beauty' hybrid selections.

Most published research on superficial scald of apples has focused on the effect of treatments on scald associated volatiles such as α-farnesene, CTs and MHO. Furthermore, recent work on DCA has only focused on its feasibility to control superficial scald. No information about the mechanisms used by DCA to maintain fruit quality is available. In this study, we hypothesize that DCA inhibits scald and maintains postharvest quality by retarding the loss of antioxidants and phytochemical properties of the fruit tissue, thereby reducing ROS accumulation and lipid peroxidation. Therefore, the objective of this study was to assess the evolution of antioxidant capacity, phytochemical contents and reactive oxygen species in

DCA stored 'Granny Smith' apples harvested at pre-optimal and optimal maturity over two growing seasons.

2. Materials and methods

2.1 Fruit source and treatments

The study was carried out over two seasons during 2013 and 2014 (referred to as season 1 and season 2, respectively) apple growing season. Seventeen-year old 'Granny Smith' apple trees grafted into M109 rootstock grown on a commercial orchard in Grabouw, South Africa (34° 12'12" S, 19° 02'35" E) were used in this study. The tree spacing was 4 x 1.5 m giving a total of 1667 trees per hectare. All trees were irrigated by micro sprinklers and pruned to a central leader. The trees received the same irrigation and fertilizer program. Fruit free from blemishes were hand-picked at 165 and 172 days after full bloom (DAFB) (which are commonly considered in the fruit industry as pre-optimal and optimal maturity periods, respectively) and transported to the laboratory in an air-conditioned car. Uniformly sized fruit with diameter of 70±2 mm and mass of 160±5 g were randomly divided into 3 replications, each comprised of 100 fruit. Fruit was thereafter stored in cold storage. The chlorophyll fluorescence non-destructive monitoring system (HarvestWatch, Satlantic Inc, Halifax, Canada) with an ability to predict and indicate the low oxygen limit (LOL) was used to determine DCA set points (Prange et al., 2011; Wright et al., 2012). In this study, the DCA was established within 48 h after harvest, using compressed air and CO_2 plus N_2 from a membrane generator (Isosep, Isolcell, Italy). Accordingly, the gas composition of the storage chamber was analysed at 90 min intervals and adjusted when necessary. Generally, the O_2 levels ranged between 0.3% to 0.5% whilst CO_2 was maintained at 1% and 95% RH. DCA storage regimes ranged between 5 d to 20 w. For each treatment and storage time, 10 fruit per replicate were peeled under subdued light. The peel was immediately frozen with liquid nitrogen, freeze dried, pulverised and stored at -80 °C until use for extraction and measurement of total antioxidants, total phenolics, ascorbic acid and lipid peroxidation. ROS production was determined from fresh samples.

2.2 Confocal microscopic analyses of ROS production

Determination of ROS in apple peel was carried out as described by Macarisin et al. (2007) and Sabban-Amin et al. (2011). The fluorescent probe 2,7-dichlorodihydrofluorescein

diacetate in which dichlorodihydrofluorescein (DCF) fluorescence measurement quantifies general oxidative stress was used. 2,7-dichlorodihydrofluorescein enters cells in the diacetate form (H_2DCF-DA), and the acetate form (H_2DCF) is hydrolyzed by intracellular esterases and then reacts with oxidants, resulting in the highly fluorescent DCF. Acetate detects a broad range of oxidizing molecules rather than a single ROS form, and it is efficient in localizing ROS within plant cells (Joo et al., 2005). Immediately before microscopic analysis, slices of apple peel were cut from fruit and immediately immersed in a small Petri dish containing 10 mL of 10.0 µM H_2DCF-DA in loading buffer (50 mM MES buffer, pH 6.5). The H_2DCF-DA was freshly prepared from a 20 mM stock solution in dimethyl sulfoxide (DMSO). To prevent light-inducible oxidation, the slices were kept in the dark for 10 min and were thereafter transferred to a new Petri dish containing loading buffer to wash off excess dye. Model IX 81 inverted confocal laser-scanning microscope (FLUOVIEW 500, Olympus, Japan) equipped with a 488 nm argonion laser was used for sample examination and image acquisition. The fluorescent probe was excited with a 488 nm laser beam and the emission was collected through a BA 515–525 filter. For autofluorescence, a BA 660 IF emission filter was used. Magnification was increased by focusing the scanning laser beam onto a smaller area of the tissue. The transmitted-light images were obtained with Nomarski differential interference contrast (DIC) optics. The relative intensity of the fluorescence signal was estimated by calculating average pixel intensity from each successive focal plane of the apple peel slice, in 5 µm steps, with MICA software (Multi-Image Analysis, CytoView, Israel). The value of fluorescence intensity presented is the mean (± standard error (SE)).

2.3 Lipid peroxidation

Malondialdehyde (MDA), a suitable biomarker for lipid peroxidation in plant tissues (Katsuhara et al., 2005; Lu et al., 2014), was quantified according to Dhindsa et al. (1981) and Siboza et al. (2013) with slight modifications. Briefly, 0.1g of freeze dried and pulverised apple peel was homogenised with 10 mL of ice cold 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 17764 g for 15 min at 4ºC to precipitate particulates. In triplicates, 1 mL aliquot of the supernatant was thoroughly mixed with 4 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was incubated at 95°C for 30 min and thereafter quickly cooled in an ice bath. After centrifugation at 10000 for 15 min at 4°C, the absorbance of the supernatant was read at 532 nm and corrected for nonspecific

absorbance at 600 nm using UV-Visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). The concentration of MDA was calculated using an extinction coefficient (∈) of 155 mM^{-1} cm^{-1}.

2.4 Phytochemical properties and antioxidant capacities

2.4.1 Sample preparation

Different apple peel extracts were prepared depending on analysis. For total phenolics and FRAP assays, 50% methanol was used whilst 1% metaphosphoric acid for ascorbic assay and distilled water for DPPH assay were used as extraction solvents. Briefly, 1 g of pulverized apple peel was accurately weighed in centrifuge tubes using a Mettler Toledo digital balance (±0.01g) followed by the addition of 10 mL of the extraction solvents. The mixture was vortexed for 30 sec before being cold-sonicated for 10 min. The sample was thereafter centrifuged at 17764 g for 15 min at 4ºC to precipitate particulates. The extracts were carefully collected into test tubes and cold stored at 4ºC.

2.4.2 Ascorbic acid content

Ascorbic acid content was quantified according to Fawole et al. (2012) with slight modifications. The mixture was vortexed for 30 sec before being ice-sonicated for 3 min, and thereafter centrifuged at 17764 g for 15 min at 4ºC. In triplicates, the extract (1 mL) was mixed with 9 mLof 2, 6-dichlorophenolindophenol dye (0.0025%). To ensure that only ascorbic acid is measured, the absorbance of the mixture was read at 515 nm within 30 min of incubation in dark environment (Barros et al. 2007). Ascorbic acid content was calculated using the calibration curve of authentic L-ascorbic acid (0.01–0.1 mg mL^{-1}), and the results were expressed as ascorbic acid equivalent (AAE) per grams dry matter (mg AAE mg g^{-1}DM).

2.4.3 Determination of total phenolics

Total phenolic (TP) content in peel methanolic extracts was determined using Folin-Ciocalteu (Folin C) colourimetric method as described by Makkar et al. (2007) and Fawole et al. (2012) with slight modifications. In triplicates, 450 µL of 50% methanol and 50 µL of extract were mixed with 1 N Folin-Ciocalteu reagent followed by 2% sodium carbonate. TP concentrations were determined spectrophotometically at 725 nm after 10 min of incubation

in the dark. Gallic acid was used as a standard and results were expressed as mean ± S.E (milligrams) of Gallic acid equivalents (mg GAE g^{-1}DM).

2.4.4 Antioxidant capacity

2.4.4.1 Ferric reducing antioxidant power (FRAP) assay

Antioxidant capacity was determined by the FRAP assay of Benzie and Strain (1996) with slight modifications. The FRAP assay measures the ability of antioxidants in the sample to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to the blue colored ferrous form (Fe^{2+}) that absorbs light at 593 nm (Khanizadeh et al. 2008). In triplicates, 150 µL of the methanolic extract was mixed with 2850 µL of FRAP reagent (300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution, 20 mM ferric chloride) and incubated in the dark for 30 min. Absorbance at 593 nm was measured using a UV-vis spectrophotometer. Antioxidant capacity was expressed as mean ± S.E (micromoles) of trolox equivalents per milligram of dry matter (µM TE mg^{-1}DM).

2.4.4.2 DPPH radical scavenging activity

Peel extract sample was tested against a stable DPPH solution according to Wong et al. (2006) with some modifications. Briefly, a 0.1 mM solution of DPPH in methanol was prepared. The initial absorbance of DPPH in methanol was measured at 515 nm and did not change throughout the assay period. In triplicates, 15 µL of the extract was mixed with methanol (735 µL) and subsequently with DPPH solution (750 µL, 0.1 mM). The change in absorbance at 515 nm was measured after 30 min incubation. Antioxidant capacity based on the DPPH free radical scavenging ability was expressed as mean ± S.E (millimolar) of ascorbic acid equivalent per milligram of dry matter (mM AAEmg^{-1}DM).

3. Statistical analysis

The results were presented as mean (±S.E) values. Analysis of variance (ANOVA) was carried out using STATISTICA 12 (StatSoft, Inc. Oklahoma, USA) according to Duncan's multiple range test. All the data was analysed and subjected to principal component analysis (PCA) and Pearson correlation. Graphical data presentations were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc. San Diego, USA).

4. Results and discussion

4.1 Reactive oxygen species

High ROS levels were observed after 20 w in cold storage as green fluorescence in the RA stored fruit. DCA stored fruit was resembled by less fluorescence and the increase in ROS level was observed after 20 weeks (Fig. 1A-F). ROS levels were much lower at 12 and 16 w in DCA stored fruit (Fig. 1B & D). ROS levels after cold storage were expressed as fluorescence units. The increases in ROS levels after 12, 16 and 20 w in cold storage were 10 to 50 fold higher in RA than in DCA stored fruit (Fig. 2). In contrast, ROS levels as reflected by fluorescence images were significantly lower in DCA throughout the entire storage period (Fig. 1B, D and F). Although DCA stored fruit showed a substantial ROS accumulation after 20 w, ROS levels were significantly ($p<0.05$) lower compared to RA stored fruit. The lower ROS levels in DCA stored fruit correlated with high postharvest quality in this fruit.

These findings corroborated with Shabban-Amin et al. (2011) who reported low ROS levels in 'Granny Smith' apples exposed to 0.5% oxygen for 10 d prior to 24 w cold storage. Interestingly, ROS levels increased with storage time in both RA and DCA cold storage. In contrast, Shabban-Amin et al. (2011) observed a decline in ROS levels with increasing storage time in 'Granny Smith' apples treated with low oxygen levels. These results suggest that the high fruit quality in DCA storage could be linked to reduced ROS levels in these storage conditions. This is attributed to the fact that scald incidence is strongly linked to ROS levels in 'White Angel x Rome Beauty' apples (Rao et al., 1998). Zubini et al., (2007) and Lu et al. (2014) also reported a strong relationship between poststorage fruit quality and ROS levels in 'Granny Smith' and 'Fuji' apples. The common ROS and derivatives responsible for oxidative stress and poor fruit quality include singlet oxygen ($^1O_2$), hydroxyl radical (OH•), superoxide radical (O_2•) and hydrogen peroxide (H_2O_2) (Racchi, 2013). Based on these results, it could be argued that the low oxygen levels in DCA created conducive environment for scavenging singlet oxygen, which induces a unique oxidative stress by directly attacking electron-rich compounds (Oh et al., 2006).

4.2 Lipid peroxidation

Membrane stability plays a critical role in the storage life of apples. In fact, previous research has shown that reduction in cell turgor through increased membrane permeability

could be partially responsible for firmness loss during storage (Lyons, 1973; Stow, 1993). The loss of structural organisation within the cells brought by membrane deteriorative processes significantly affects fruit quality and its market value. Therefore, postharvest treatments that maintain and protect membrane lipids from solidification and peroxidation are highly beneficial to fruit quality and shelf-life (Lafuente et al., 2005). This is due to the fact that membrane is the primary site for oxidative stress and damage during senescence and ripening (Rao et al., 1998).

In this study, an interaction among harvest maturity, storage time, storage condition (A*C*D; p=0.0068) and growing season, storage time, storage condition (B*C*D; p=0.0032) highly significant influence on lipid peroxidation. This interaction could be explained by rapid increase of lipid peroxidation, expressed as MDA concentration, in RA with prolonged storage time (Fig. 3A). The increase was more pronounced in RA stored fruit compared to those stored under DCA (Fig.3A). Fruit stored in DCA was characterized by lower levels of lipid peroxidation. These results are contrary to Bordonaba et al. (2013) who found no differences in 'Granny Smith' apples stored in convectional CA and ultra-low oxygen levels. Interestingly, Shabban-Amin et al. (2011) reported a significantly lower electrolyte leakage in 'Granny Smith' apples stored at low oxygen conditions (0.5% O_2). The lipid peroxidation in DCA stored fruit was similar to that found in 'Gala' apples showing low lipid peroxidation and high fruit quality after heat treatments before storage at 0 °C for 8 w (Shao et al., 2012).

Notably, fruit harvested at optimal maturity appeared to have higher lipid peroxidation compared to fruit harvested at pre-optimal maturity. Moreover, the effect of seasonal variation seemed to be dependent on storage condition. Under DCA, season 1 had higher lipid peroxidation, however, in RA stored fruit season 2 had higher lipid peroxidation (Fig. 3B). It could be argued that a membrane from optimally harvested 'Granny Smith' apples is more sensitive to oxidative stress. This notion would be in agreement with Rao et al. (1998) who suggested that membrane is the primary biochemical site for senescence and ripening. The lower fruit firmness associated with advanced fruit maturity could also be linked to reduced membrane stability. However, the opposite has been reported in other fruits. For instance, pre-optimally harvested Mango fruit exhibits high lipid peroxidation and consequently high chilling injury and inferior fruit quality (Zhao et al., 2009). Hypothetically, fruit harvested at pre-optimal maturity should have high lipid peroxidation; this is attributed to the fact that this kind of fruit is highly susceptible to postharvest physiological disorders.

The higher lipid peroxidation in optimally harvested, rather than pre-optimally fruit observed in this study indicates the complexity of biochemical and physiological processes involved at postharvest.

4.3 Antioxidants capacity

Antioxidants are responsible for scavenging ROS during storage and consequently improve postharvest quality of fruit and vegetables. High storage quality in apples is generally associated with high antioxidant capacity (Barden and Bramlage, 1994a). Therefore, postharvest storage conditions and treatments should either maintain or improve the antioxidant pool in stored fruits. In this study, an interaction of harvest maturity, storage time and storage condition (A*C*D) had a significant influence on antioxidant capacity as measured by DPPH ($p<0.0001$) and FRAP assay ($p=0.0030$). Hydrophilic and lipophilic antioxidant capacity as quantified by FRAP and DPPH assay appeared to reduce during storage particularly in RA stored fruit (Fig. 4A and B). This trend corresponds with inferior fruit quality in RA storages. This result corroborates with Callerani et al. (1990) who reported that lipophilic antioxidants decrease during storage. Previous research has shown that postharvest treatments such as 1-MCP or heat treatments that are used to prolong storage life also increase antioxidant capacity. For instance, Shaham et al. (2003) showed that unlike untreated fruit, 1-MCP or heat treated 'Granny Smith' apples had higher lipophilic and hydrophilic antioxidant capacity and consequently superior fruit quality fruit at the end of 16 w storage at 0 °C. In contrast, Leja et al. (2003) found no significant difference in 'Jonagold' and 'S'ampion' apples stored in CA (2% O_2; 2% CO_2) when compared to those stored in RA. Based on these results, it could be argued that the high quality in DCA stored fruit is related to high antioxidant capacity enabling the scavenging of ROS during storage.

Fruit harvested at optimal maturity appeared to have higher antioxidant capacity. This result is in agreement with the notion that harvest maturity significantly affects antioxidant capacity (Refer to chapter 1). The reduction in hydrophilic antioxidant capacity in optimally harvested fruit during storage is similar to that found in 'Cortland' and 'Delicious' apples (Barden and Bramlage, 1994b). At harvest, optimally harvested fruit had higher antioxidant capacity (both lipophilic and hydrophilic) compared to pre-optimally harvested fruit; however, this difference did not persist throughout the storage. Barden and Bramlage (1994b) also found a similar trend in 'Cortland' apples stored at 0 °C for 20 w. It could be argued that the reduced hydrophilic antioxidant capacity in optimally harvested fruit is linked to their

high active state to scavenge ROS during stress. Further research is warranted to investigate this possibility.

4.4 Total phenolics

Total phenolics play an intrinsic role in extending the storage and shelf-life of apples. Previous research has shown that fruit which is resistant to physiological disorders generally has higher contents of phenolics when compared with susceptible cultivars. For instance, Ju and Bramlage (1999) reported a higher phenolic concentration in scald resistant 'Golden Delicious' apples whilst a lower phenolics concentration was reported on scald susceptible 'Cortland' and 'Empire' apples. Total phenolics are highly involved in inhibiting ROS such as α-farnesene accumulation during storage (Ju and Bramlage, 2000). In this present study, an interaction between fruit maturity, storage condition and growing season (A*B*D; p=0.0225) had a significant effect on total phenolic content making it hard to sensibly interpret the results. However, it was clear that compared to RA, DCA stored fruit maintained higher total phenolic content (Fig. 5A). Moreover, fruit harvested at pre-optimal maturity appeared to higher total phenolic content compared to those harvested at optimal maturity. This difference was however less pronounced in DCA stored fruit. Another interaction among harvest maturity, storage time and storage condition (A*C*D; 0.0054) had a significant effect on total phenolics. Throughout the storage time, total phenolics appeared to be higher pre-optimally harvested fruit under RA storage. Storing apples in DCA clearly reduced the loss of total phenolic contents (Fig. 5B). This finding is consistent with other quality retaining technologies such as the application of ethephon before harvest which has been reported to increase cuticular phenolics and consequently increase storage potential (Ju and Bramlage, 2000). The effect of DCA on specific phenolic compounds and their pathways should be evaluated. This might be useful in improving DCA technology and having a deeper understanding of scald development.

4.5 Ascorbic acid

Ascorbic acid is one of the major antioxidants and ROS scavenger involved in wide aspects of cellular defence metabolism against biotic and abiotic stress in fruit (Smirnoff et al., 2001; Davey et al., 2007). In fact, Barden and Bramlage (1994a) found a strong correlation between ascorbic acid concentrations and scald resistance in 'Cortland' and 'Delicious' apples. High incidence of brown heart is highly correlated to ascorbic acid loss in

'Conference' pears (Eccher Zerbini et al., 2002). In this present study, a strong interaction between harvest maturity and growing season (A*B; p=0.0162) had a significant effect on ascorbic acid concentration. For fruit harvested at pre-optimal maturity, ascorbic acid concentration was lower in season 1 compared to season 2 (Fig. 6A). In contrast, optimal maturity fruit had higher ascorbic acid concentration in season 1 compared to season 2. Overall, fruit harvested at optimal maturity had higher ascorbic acid concentration compared to those harvested at pre-optimal maturity. Fruit maturity has a significant influence on ascorbic acid concentration of pome fruits. In this present study, optimally harvested fruit had higher ascorbic acid concentrations. This result is contrary to Barden and Bramlage (1994b) who reported a strong negative correlation between harvest maturity and ascorbic acid concentration in 'Cortland' and 'Delicious' apples. Ascorbic acid concentration reduced with advancement in fruit maturity (Barden and Bramlage, 1994b). Eccher Zerbini et al. (2002) also found that advanced fruit maturity increase the rate of ascorbic acid loss in 'Conference' pears. The high ascorbic acid in optimally harvested fruit as found in this study could be due to the difference in physiological and biochemical properties of cultivars. Based on these findings, the poor fruit quality associated with pre-optimally harvested fruit (Anet, 1974) could be linked to lower ascorbic acid and less efficient antioxidant defence system.

Another strong interaction between storage time and storage condition (C*D; p=0.0010) had an effect on ascorbic acid concentration. In RA stored fruit, ascorbic acid concentration decreased during storage (Fig. 6B). Interestingly, DCA stored fruit had higher concentrations of ascorbic acid compared to RA stored fruit. Based on this result, it could be argued that the low fruit quality in RA stored fruit corresponds to low ascorbic acid concentrations in these storage conditions. This result showed that the ability of low oxygen (<0.5% O_2; 1 CO_2) levels to reduce ascorbic acid loss during storage might had a beneficial effect on fruit quality. Contrary to this result, Franck et al. (2003) observed a low ascorbic acid concentration in CA (2.5% O_2; 0.7% CO_2) stored 'Conference' pears when compared to RA. In their study, it was concluded that very low CO_2 reduces ascorbic concentration. To the best of our knowledge, this is the first study to evaluate the evolution of antioxidants and phytochemicals in DCA stored fruit.

4.6 Principal component analysis

Data was subjected to principal component analysis (PCA) to obtain a broader view of biochemical changes taking place in DCA and RA stored fruit over two growing seasons.

PCA scores and loadings are shown in Fig. 7 and Table 1. Clear separation was observed demonstrating the effects of storage conditions on the metabolic, antioxidant and phytochemical properties in 'Granny Smith' apples. The first two PCs explained 84.83% (F1= 69.96%; F2= 14.87%) of the total variance and clearly separated fruit stored in DCA or RA. PCA showed two distinct clusters for DCA and RA stored fruit.

Eigen values and factor loadings showed major differences in fruit stored at DCA and RA (Table 1). Liu et al. (2003) mentioned that the classification of factor loadings is considered strong, moderate and weak when corresponding to loading values of 0.75, 0.75-0.50 and 0.50-0.30, respectively. In both growing seasons, positive scores of F1 corresponded with RA stored fruit whilst DCA stored fruit had high negative scores along F1. The scores can be interpreted by factor loadings (Table 1), with F1 showing strong negative correlations for DPPH, FRAP, total phenolics and ascorbic acid, and negative correlations for lipid peroxidation and ROS. This study showed that DCA stored fruit is characterized by higher antioxidant capacity, total phenolics and ascorbic acid concentration whilst RA stored fruit has higher lipid peroxidation and ROS concentration. The short distances between total phenolics, ascorbic acid and antioxidant capacity shows the significant contribution of phenolics and ascorbic acid to antioxidant capacity as quantified by both FRAP and DPPH assay; a trend that has previously been reported in pomegranate fruit (Fawole and Opara, 2013).

To elucidate the relationship between the metabolic, antioxidant and phytochemical components for both DCA and RA stored fruit, Pearson correlation was performed (Table 2). Strong and significant ($p<0.05$) relationships among some of the biochemical properties were revealed. For instance, antioxidant capacity (DPPH) had a strong positive correlation with ascorbic acid (r= 0.759) whilst a positive and medium correlation (r= 0.629) existed between total phenolics and antioxidant capacity (FRAP). This finding suggests that antioxidant capacity in 'Granny Smith' apples could be depended on total phenolics and ascorbic acid concentration. Another interesting relationship was a medium positive correlation between lipid peroxidation and ROS. This correlation supports the evidence that ROS is responsible for membrane damage in fruit (Katsuhara et al., 2005; Lu et al., 2014). Interestingly, lipid peroxidation had a strong negative correlation with total phenolics (r= -0.677), antioxidant capacity (r= -0.701; r= -0.836) as measured by DPPH and FRAP assay, respectively. This

result suggests that postharvest treatments maintaining antioxidant capacity of apples will retard ROS accumulation and consequently reduce membrane damage.

5. Conclusion

The influence of DCA and seasonality on metabolic and antioxidant properties at pre-optimal and optimal maturity of 'Granny Smith' apples was investigated. Results obtained have shown major metabolic and biochemical changes in DCA and RA stored. This study has demonstrated that the high quality fruit associated with DCA is linked to reduced loss of antioxidants. This enables fruit to maintain lower levels of ROS and consequently, membrane damage. Although fruit maturity influences the antioxidant capacity of fruit, this study has shown that DCA has an ability to maintain high antioxidant capacity regardless of fruit maturity.

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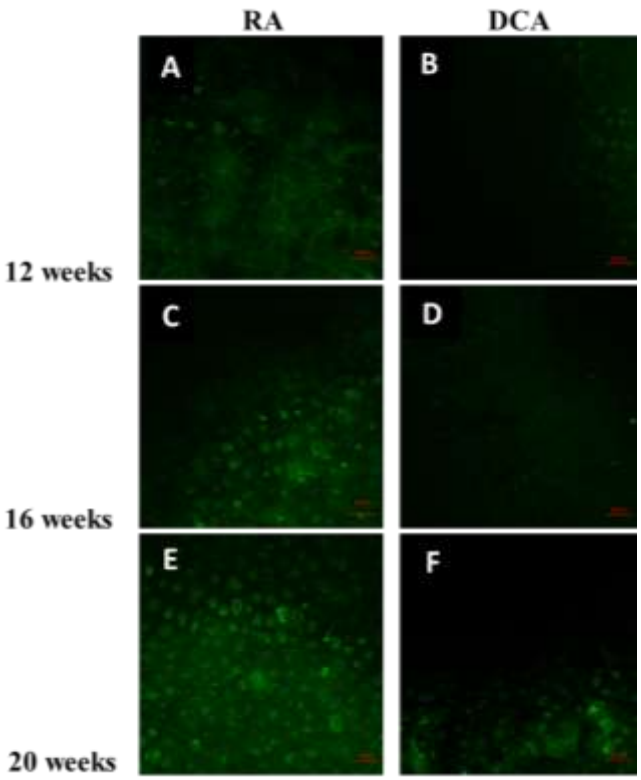

Fig. 1. Confocal laser-scanning fluorescence images of 'Granny Smith' apple peel. RA (A, C and E) and DCA stored (B, D and F) after 12, 16 and 24 weeks in cold storage at 0 °C. Images are projections of several optimal sections collected by confocal microscopy, showing DCF fluorescence. Apple peel slices were stained with H_2DCF-DA and viewed under constant-excitation light intensity.

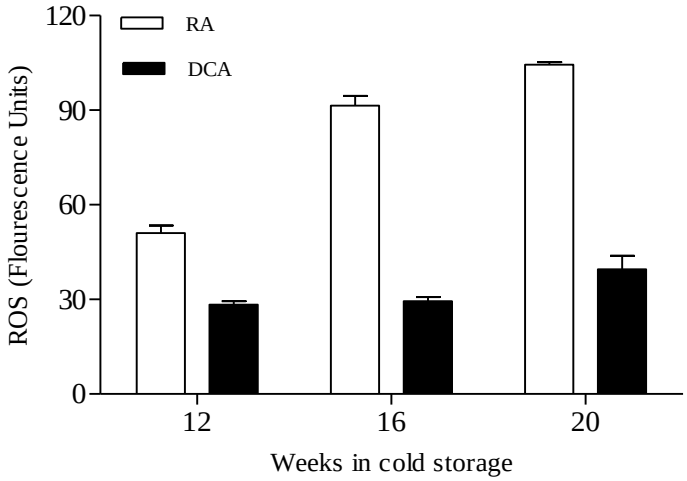

Fig. 2. Production of reactive oxygen species (ROS), as quantified by relative intensity of dichlorodihydrofluorescein diacetate (DCF) fluorescence in 'Granny Smith' apple peel. Data points with different letter(s) differ significantly according to Duncan's multiple range test ($p<0.05$).

Table 1: This table shows the significance levels of the factors and their interactions of the figures presented below

Source	Significance level				
	MDA	**DPPH**	**FRAP**	**Total Phenolics**	**Ascorbic Acid**
Maturity (A)	<0.0001	0.0570	<0.0001	0.0048	<0.0001
Season (B)	0.1397	0.4381	0.4304	0.0127	0.7146
Storage Time (C)	<0.0001	<0.0001	<0.0001	<0.0001	0.0002
Storage Condition (D)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
A*B	0.0629	0.0052	<0.0001	0.9326	**0.0162**
A*C	<0.0001	<0.0001	0.0098	<0.0001	0.6388
B*C	0.0990	0.5840	0.0410	0.3616	0.4198
A*D	<0.0001	0.0091	0.0032	0.2678	0.5752
B*D	<0.0001	0.7915	0.0078	0.9281	0.9386
C*D	<0.0001	<0.0001	<0.0001	0.0009	**0.0010**
A*B*C	0.0602	0.0513	0.9188	0.1497	0.2117
A*B*D	0.4992	0.7996	0.4046	**0.0225**	0.2285
A*C*D	**0.0068**	**<0.0001**	**0.0030**	**0.0054**	0.8954
B*C*D	**0.0032**	0.7653	0.7207	0.6444	0.7519
A*B*C*D	0.3372	0.6236	0.9705	0.1607	0.3296

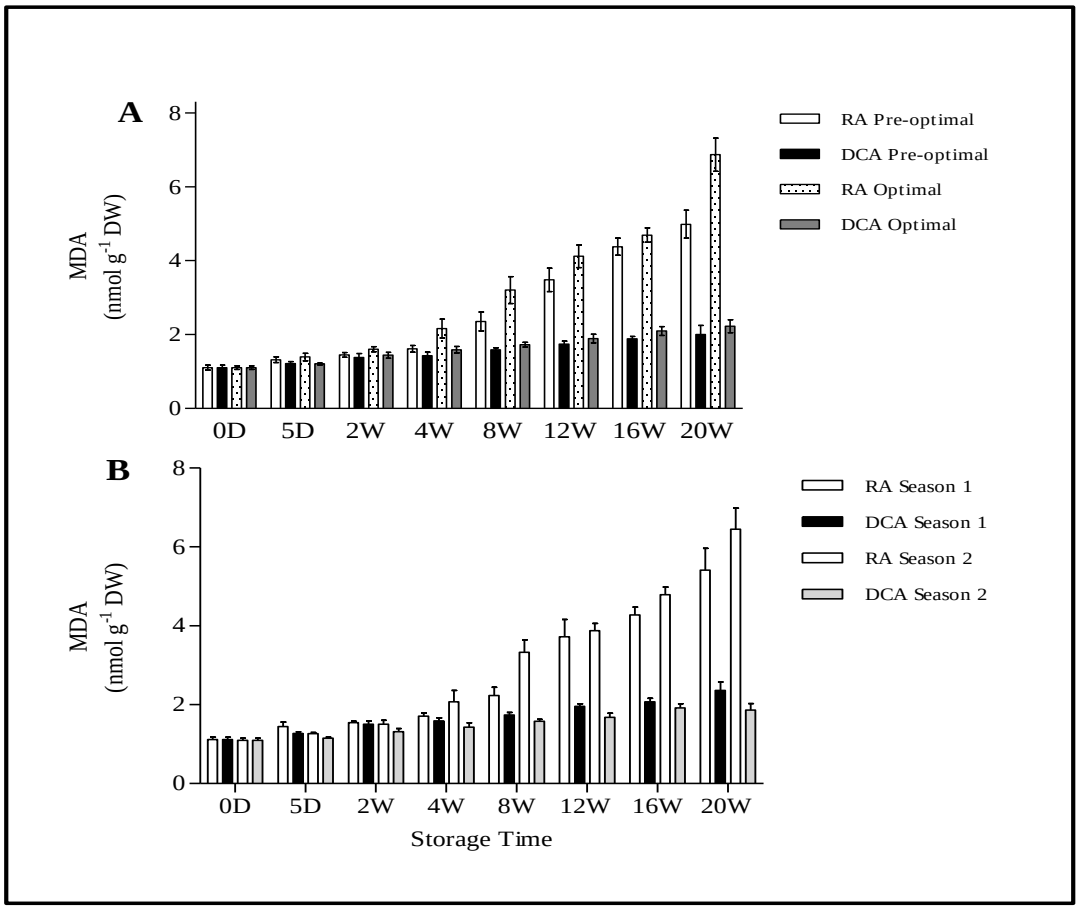

Fig. 3. Changes in malondialdehyde (MDA) concentration of 'Granny Smith' apples during RA and DCA cold storage at 0 °C as influenced by harvest maturity*storage time*storage condition (A) and growing season*storage time*storage condition (B) growing season. Each value shown represents the average result and standard error (SE).

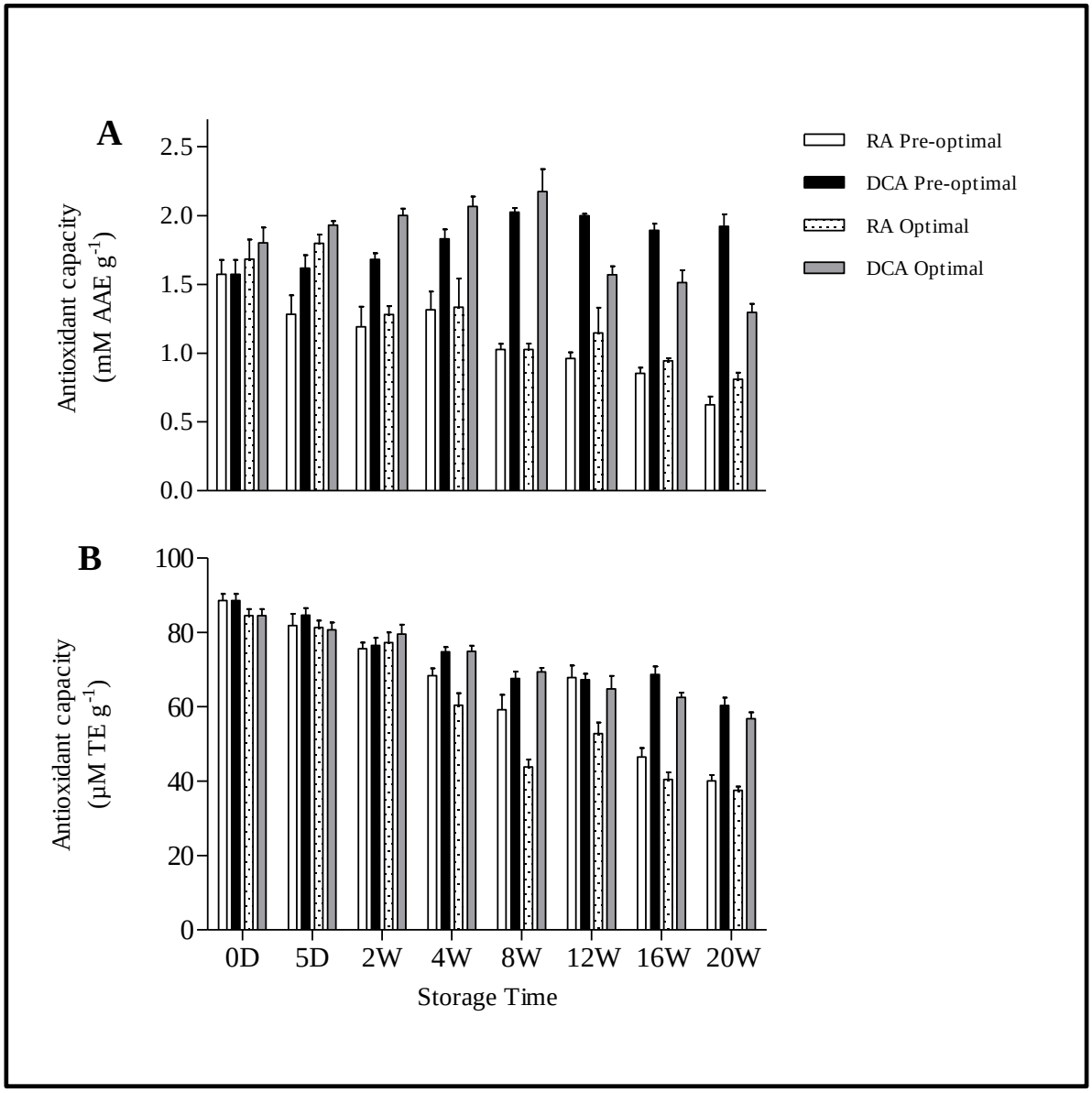

Fig. 4. Antioxidant capacity as quantified by DPPH (A) and FRAP assay (B) of 'Granny Smith' apples during RA and DCA cold storage at 0 °C. Each value shown represents the average result and standard error (SE).

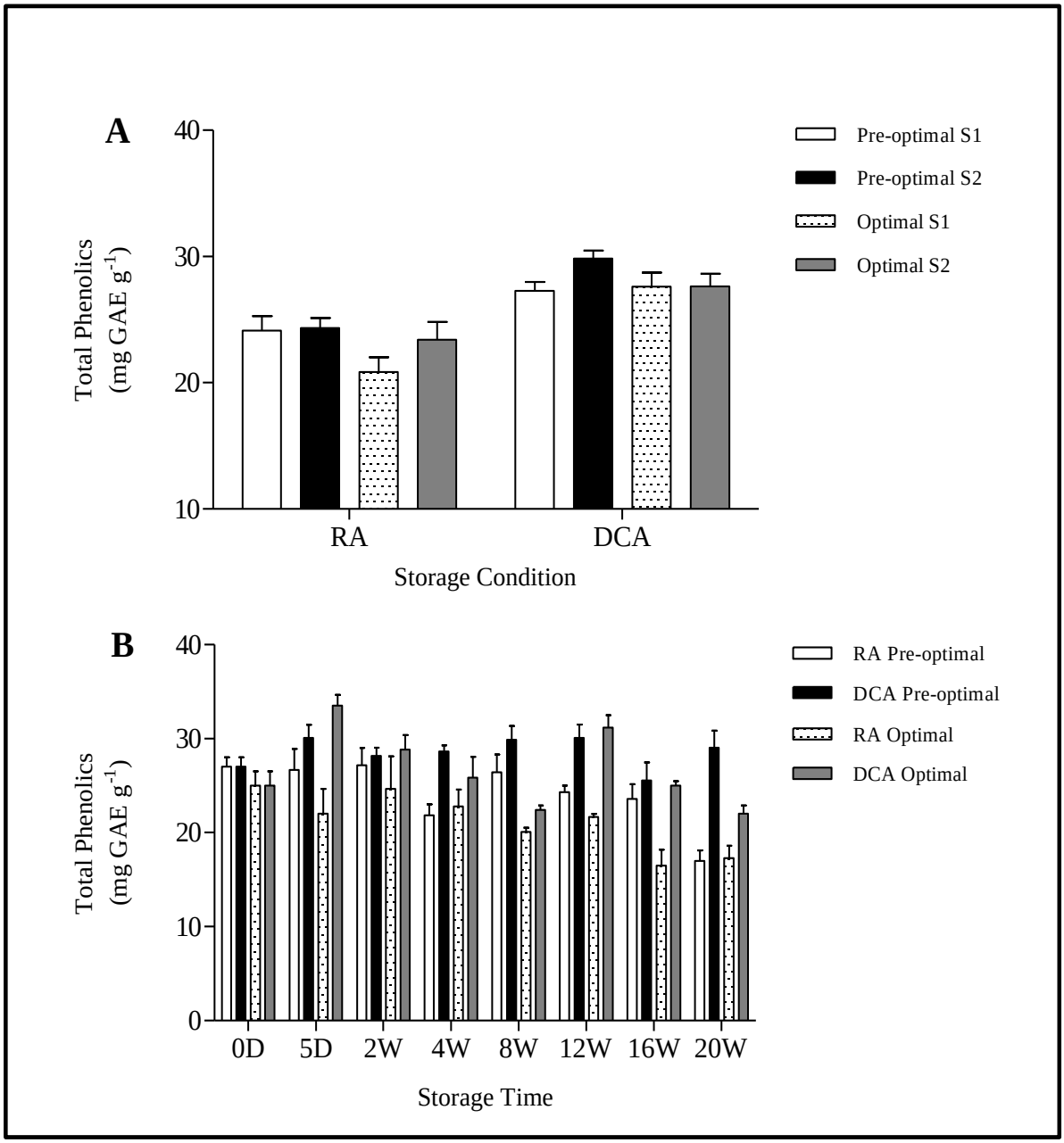

Fig. 5. The effect of storage condition*harvest maturity*growing season (A) and harvest maturity*storage time*storage condition (B) on total phenolic concentration of 'Granny Smith' apples cold stored at 0 °C. Each value shown represents the average result and standard error (SE). Abbreviation: S1-season 1, S2-season 2.

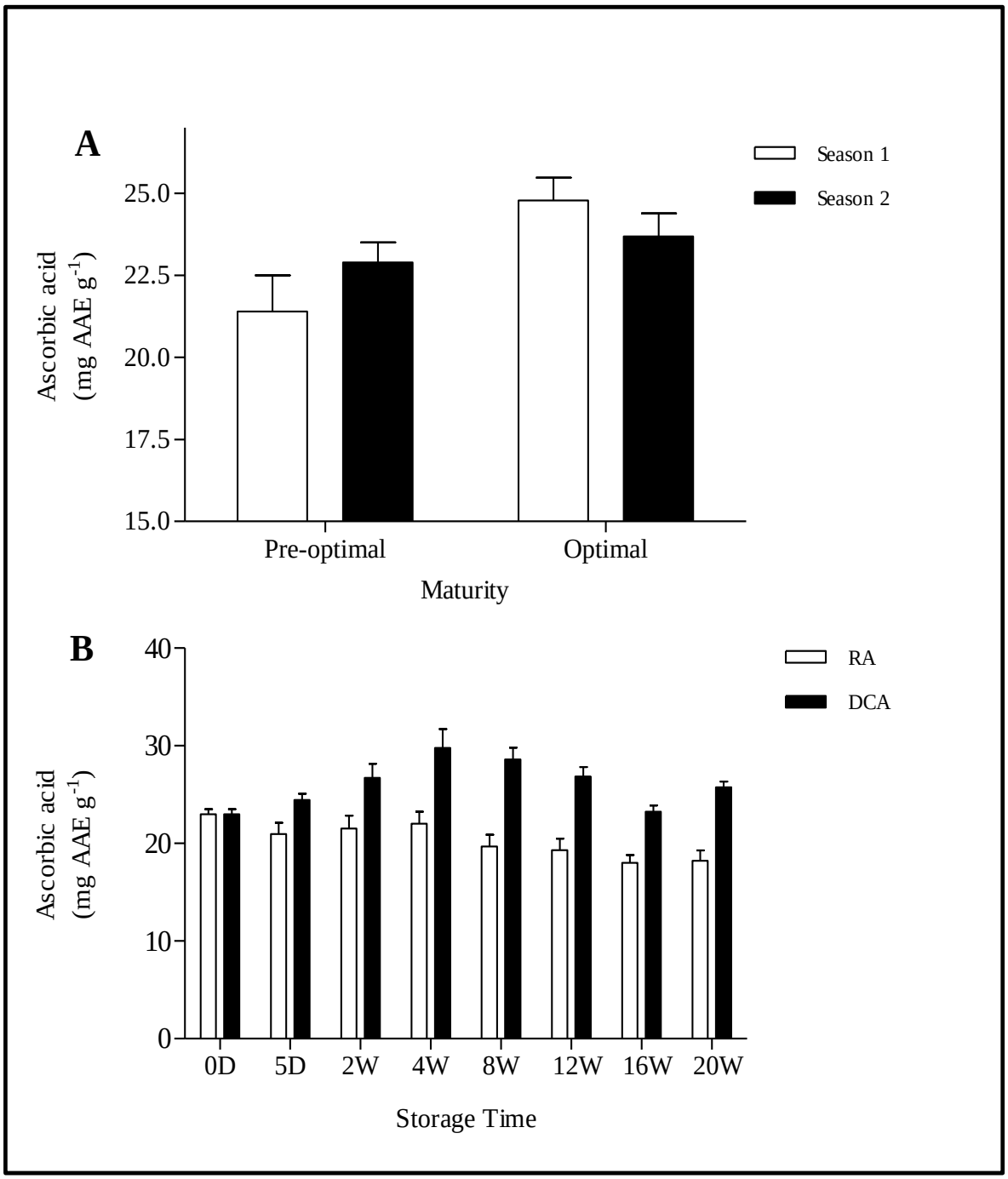

Fig. 6. The effect of harvest maturity*growing season (A) and storage time*storage condition (B) on ascorbic acid concentration of 'Granny Smith' apples cold stored at 0 °C. Each value shown represents the average result and standard error (SE).

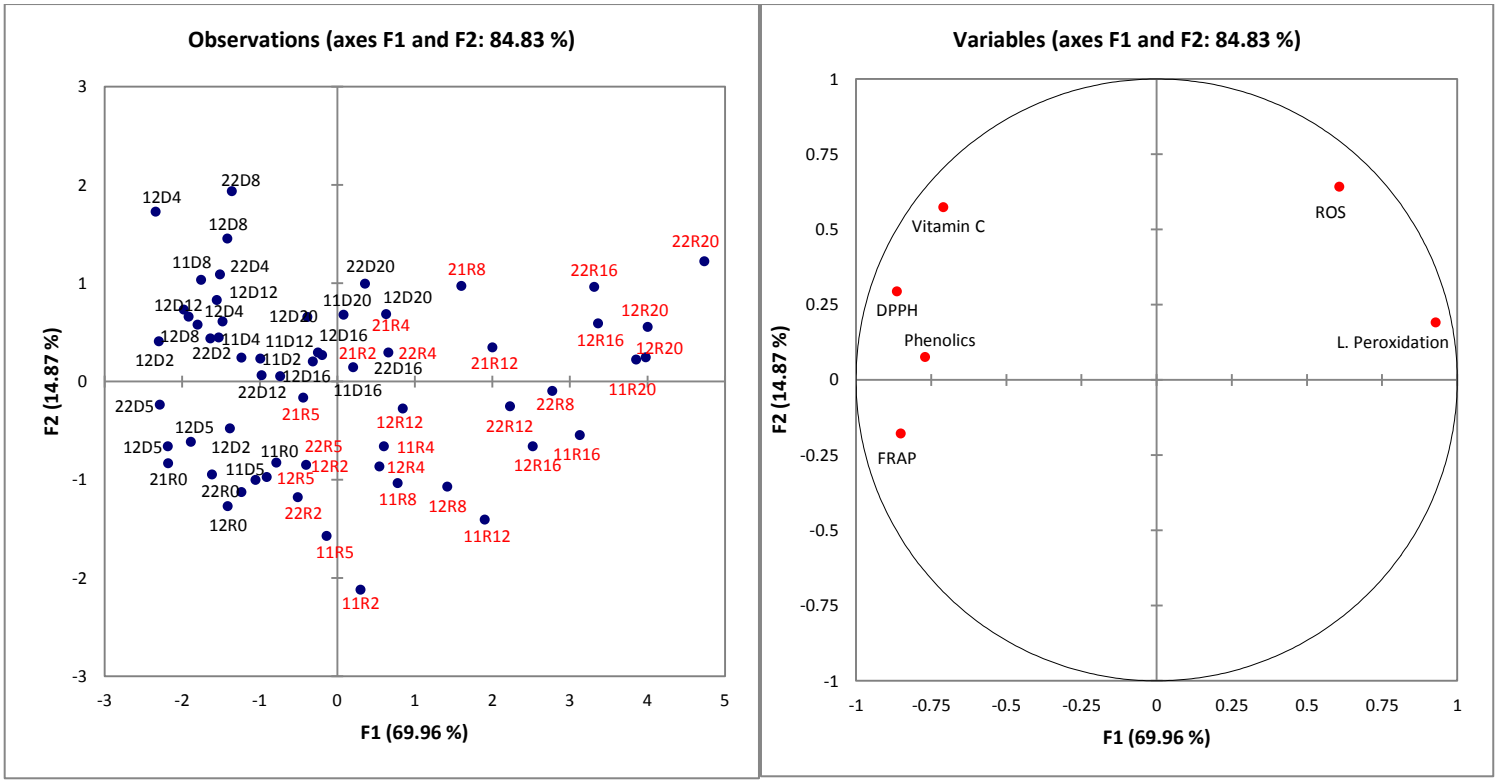

Fig. 7. Principal component analysis chart for two principal components showing correlation between measured metabolic and antioxidant properties of DCA (black) and RA (red) stored 'Granny Smith' apples.

PAPER 6

The efficacy of repeated application of dynamic controlled atmospheres on scald potential 'Granny Smith' apples

Abstract

This study investigated the influence of storage time and efficacy of repeated dynamic controlled atmospheres (DCA) applications to control superficial scald of 'Granny Smith' apples with a high potential of scald development in two growing seasons. Fruit were stored for up to 16 w in DCA with a 14 d of interruption in refrigerated air (RA) at -0.5 °C, 95% RH. Fruit was again stored in DCA. The scald potential for each storage time in each season was assessed by storing fruit in RA and used as the control, in order to ascertain the efficacy of repeated DCA treatments. Superficial scald incidence, total soluble solids, (TSS), titratable acidity (TA), ground colour, fruit firmness and ethylene production were measured. Alpha-farnesene and 6-methyl-5-hepten-2-one (MHO) production were also measured using gas chromatography (GC-MS). Principal component analysis (PCA) was used to visualize metabolic changes and the relationship among them. Results showed maximum superficial scald incidence of 1% and 99% in repeated DCA and RA, respectively. Fruit stored in DCA repeatedly was characterized by a higher fruit firmness, ground colour and titratable acidity. Ethylene, α-farnesene and MHO production were significantly lower in repeated DCA compared to only RA stored fruit. Seasonal changes had no significant effect on metabolic parameters studied. The data distribution in the PCA displayed two clusters that could easily be identified. These clusters allowed distinction between fruit stored in RA only and repeated DCA. Ethylene, α-farnesene and MHO production had a strong and positive correlation with scald incidence. This study demonstrated that repeated DCA treatments commonly used by industry can effectively control superficial scald in a high scald potential season.

Keywords: *Malus domestica*, superficial scald, α-farnesene, ethylene, 6-methyl-5-hepten-2-one

1. Introduction

Superficial scald of apples (*Malus domestica* Borkh.) is a common physiological disorder that develops during cold storage of susceptible cultivars. If not controlled, market

value of the fruit is reduced. This disorder is characterized by necrosis of the hypodermal cortical tissue (Bain, 1956) with epidermis only affected in severe injury (Lurie and Watkins, 2012). Its occurrence and severity increase with increasing storage duration. Fruit maturity and seasonal variations have significant influence on scald susceptibility (Barden and Bramlage, 1994). Generally, pre-optimal harvested fruit are highly prone to scald (Ingle, 2001). Array of biochemical mechanisms are responsible for scald development. The accumulation of ethylene, α-farnesene and its oxidation to conjugated trienols (CTs) and methyl-heptane-one (MHO) is arguably the cause of scald (Ju and Curry, 2002; Isidoro and Almeida, 2006). In addition, reactive oxygen species are also involved in auto-oxidative process that reduces membrane integrity and subsequently leading to scald (Barden and Bramlage, 1994; Watkins et al., 2000; Ingle, 2001).

Different postharvest methods have been used to control superficial scald. The use of non-chemical treatments such as proper ventilation and oil wraps (essential oils) were the first methods which proved to be effective (Brooks et al., 1923; Scott et al., 1995; Curry, 2000; Ju and Curry, 2000). Controlled atmosphere (CA) storage has been widely used in both apples and pears (Sabban-Amin et al., 2011; Wang and Dilley, 1999; Whitaker, 2000). However, all of these methods cannot completely control scald. Moreover, fruit in CA storage have high risk of off-flavours due to anaerobic respiration (Mattheis et al., 2005). The static CA cannot detect the low oxygen concentrations (above anaerobic respiration) for optimal advantage of such atmospheres (DeLong et al., 2004; Zanella et al., 2005). The inhibitory effects of 1-methylcyclopropene (1-MCP) and diphenylamine (DPA) on scald development have been reported (Dauny and Joyce, 2002; Golding et al., 2001; Jung and Watkins, 2008; Moggia et al., 2010; Rudell et al., 2005; Watkins et al., 2000; Zanella, 2003). However, health concerns have been raised regarding the use of DPA in food material (Santovito et al., 2012). In fact, several European countries reject its usage on fruit (Lurie and Watkins, 2012). 1-MCP is another effective postharvest treatment for scald control; however, chemical treatments are losing popularity in food industry particularly in organic and high paying markets. Developing non-chemical postharvest treatments that provide effective scald control is therefore essential.

Recent research findings have identified an improved version of CA termed dynamic controlled (DCA), a potential replacement for chemical methods. The DCA technology has been tested in some pears and apple cultivars. For instance, reduced scald in pears (Prange et

al., 2011) and apple cultivars such as 'Golden Delicious', 'Gala', 'Braeburn', 'Idared', 'Maigold', 'Elstar' and 'Pinova' has been reported (Gabioud et al., 2009; Mattheis et al., 1998; Raffo et al., 2009; Zanella et al., 2008). However, there are questions yet unanswered regarding DCA technology. For instance, unexpected demand of fruit and need to remove individual orchard lots necessitates the opening and resealing of cold storage facilities. Studies on 1-MCP have revealed that storage interruption do not reduce its effectiveness to maintain postharvest quality in CA stored fruit (Mattheis, 2008). However, it remains unknown if DCA stores are opened and then resealed after an extended refrigerated air (RA) period whether it is effective in controlling superficial scald in a batch that has a high potential to develop scald and whether postharvest quality can be maintained. Therefore, the objective of this study was to determine the efficacy of a repeated DCA treatment after an interruption of an RA period on scald control for various cold storage periods on scald prone fruit and its ability to maintain postharvest quality of 'Granny Smith' apples.

2. Materials and methods

2.1 Fruit source, treatments and storage

The study was carried out over two seasons during 2013 and 2014 seasons. Apple fruit (*Malus* x *domestic* Borkh.) cv. 'Granny Smith' grown in a commercial orchard were hand-picked from Valley Green Farm in Grabouw, South Africa (34° 12'12" S, 19° 02'35" E) at optimal maturity. Uniformly sized fruit with diameter of 70±2 mm and mass of 160±5 g were randomized to provide 3 replications of 100 fruit per treatment. DCA set points were determined by a chlorophyll fluorescence non-destructive monitoring system (HarvestWatch, Satlantic Inc, Halifax, Canada) which can predict and indicate the occurrence of low oxygen stress (Prange et al., 2003; Wright et al., 2012). In this study, the DCA was established within 48 h after harvest, using compressed air and CO_2 plus N_2 from a membrane generator (Isosep, Isolcell, Italy). The gas composition of the storage chamber were analysed at 90 min intervals and adjusted when necessary. The O_2 levels generally ranged between 0.3% to 0.5% whilst CO_2 was maintained at 1% and relative humidity of 95% RH.

Fruit were stored for 24 to 126 d in DCA storage. Each DCA storage regime was interrupted by 14 d RA storage simulating the sourcing period for packing that is often commercially caused by unexpected fruit demand. After the sourcing period, DCA storage conditions were re-established. Each treatment was followed by a 6 w RA period at -0.5 °C

simulating the shipment period that is often experienced by distant suppliers. Four DCA chambers with a volume of 15.65 m^3 were used. In each chamber, a sensor was installed in a plastic basket with a sample of 6 apples. To show the potential of superficial scald development on this batch of fruit, half the fruit were stored in air (-0.5 °C) for the same storage periods used for the various repeated DCA treatments.

2.2 Assessment of fruit quality

Fruit quality was evaluated at 7 d after storage at ambient conditions (20 °C and 65% RH). Fruit was assessed for the presence of disorders. Scald incidence was recorded as the number of damaged fruit (% fruit with scald symptoms). Ten randomly selected fruit per replicate were used to assess fruit quality. Ground colour was measured at two random positions along the equator of each apple using a calibrated chromameter (Minolta Chroma Meter, CR-300, Minolta, Japan). The hue angle [°H= arctan (b*/a*)] was calculated (Pathare and Opara, 2012) and used to measure ground colour (Zanella, 2003).

Fruit firmness measurements were carried out using Texture Analyser (Tensilon model UTM-4L, Tokyo Measuring Instruments Co., Ltd., Japan) with a 11.1mm compression probe. Operating conditions of the instrument were: pre-test speed 1.5 mm/s, 0.5 mm/s test speed, 10.0 mm/s post-test speed, and 0.20 N trigger force. The place where the penetrometer entered was peeled with a potato peeler. Each fruit was aligned horizontally from the stem end to the apex, on a smooth holder to prevent slipping. For each fruit two measurements on opposite sides of fruit were taken. Fruit firmness (N) of the fruit was taken as force of compression.

Total soluble solids (TSS) and titratible acidity (TA) was measured from three replications of ten fruit composite sample. Total soluble solids (ºBrix) of the juice were measured using a digital refractometer (Atago, Tokyo, Japan). Titratible acidity expressed as malic acid, was measured at room temperature using titration to an endpoint of pH 8.2, a Metrohm 862 compact titrosampler was used (Herisau, Switzerland).

2.3 Ethylene production

Ethylene was measured as described by Öz and Ergun (2009) and Öz (2011) with slight modifications. Briefly, six fruit per replicate were used to measure ethylene production.

Each fruit was weighed using an electronic balance (Mettler Toledo, Switzerland) with an accuracy of 0.01g, and thereafter enclosed in a 1 L airtight glass jar with a rubber septum for 1 h at 20 °C. An infrared ethylene analyser (ICA56 ppm, United Kingdom) was used for measurements and the results were expressed as $\mu L C_2H_2 kg^{-1} h^{-1}$.

2.4 Headspace volatile analysis

Vial headspace was analysed according to Mayuoni-Kirshinbaum et al. (2012) and Caleb et al. (2013). Apple peel was carefully removed from ten fruit per replicate (3 replicates per treatment). The peel (5 g/vial) was thereafter placed into 20 mL SMPE glass vials before adding 10 µL of 3-octanol internal standard. The vials were sealed and equilibrated at 50 °C for 10 min in CTC autosampler incubator (Agilent PAL, Agilent, Palo Alto, CA). A 50/30 µm divinylbenzene/-carboxen/-polydimethylsiloxane coated fibre was thereafter exposed to the headspace for 20 min at 50 °C. After extraction, desorption of the volatile compounds from the fibre coating was carried out in the injection port of gas chromatography-mass spectrometer (GC–MS) (Agilent 6890N, Agilent, Palo Alto, CA). The temperature of the vaporization chamber was maintained at 250 °C for the injection.

Vial headspace was analysed using GC–MS connected to a mass spectrometer detector (Agilent 5975 MS, Agilent, Palo Alto, CA). The GC–MS system was equipped with Rtx®-5Sil MS, and having a 95% polydimethyl siloxane/5% polydiphenyl siloxane stationary phase and the dimensions of the column were 30 m length, 0.25 mm inner diameter, and 0.5 µm film thickness. Helium was used as carrier gas at a flow rate of 1.2 mL min^{-1}. The oven temperature was as follows: 40 °C for 2 min; and then ramped up to 250 °C at 5 °C min^{-1} and held for 5 min. The ion source and quadropole were maintained at 240 °C and 150 °C, respectively. The transfer line temperature was maintained at 280 °C. Alpha-farnesene and MHO were identified by a library search and quantified using abundance characteristic ion 93 and 108, respectively. Generally, α-farnesene gave a single peak at 22.2 min while MHO gave a peak at 16.6 min. A reading of 10^4 in abundance of ion 93 or 108 was defined as one unit and expressed as U g^{-1} (Ju and Curry, 2000).

3. Statistical analysis

The results were presented as mean (±S.E) values. Analysis of variance (ANOVA) was carried out using STATISTICA 12 (StatSoft, Inc. Oklahoma, USA) according to

Duncan's multiple range test. Principal component analysis (PCA) was performed using statistical software XLStat, version 7.5.2 (Addinsoft, New York, USA). Graphical data presentations were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc. San Diego, USA).

4. Results and discussion

4.1 Superficial scald

Superficial scald development in 'Granny Smith' apples was reported as percentage of scalded fruit. The storage condition had a significant ($p<0.0001$) effect on superficial scald incidence (Table 1). In both seasons the fruit used had the potential to develop high levels of scald (>50%) after 42 d of storage and ripening as shown by the RA stored fruit. For these storage times, superficial scald incidence was effectively inhibited with repeated DCA treatment and only after 126 d total storage time plus 7 d ripening at 20 °C, 1 and 2 % of scald developed for fruit harvested in 2013 and 2014, respectively. At that point, the fruit had the highest potential for scald development as all RA stored fruit were affected as recorded (Table 1). Repeated DCA application with an interruption of 14 d under RA for sourcing and packing was therefore effective in controlling scald in fruit with a very high potential to develop scald after shelf-life. Growing seasons had a significant effect on superficial scald incidence. In the 2013 season, scald incidence was significantly higher than 2014 season evident from RA stored fruit after 42 and 70 d of storage (Table 1). The repeated DCA treatments with an intermittent break of 14 d stored for 42 and 70 d had similar results to the findings of Mattheis and Rudell (2011) who reported a complete control of scald incidence in DCA stored 'd'Anjou' pears that did not have a break and is therefore a comparable treatment in terms of efficacy. The potential of low oxygen storage to control scald has previously been reported. For instance, Wang and Dilley (2000) reported low scald incidence in 'Granny Smith' apples exposed to initial low oxygen stress prior CA storage. Moreover, studies by Pesis et al. (2010) showed that 7-10 d low oxygen stress treatments at 20 °C before cold storage in air reduced scald incidence.

4.2 Total Soluble Solids (TSS) and Titratible Acidity (TA)

Changes in TSS and TA during storage are shown in Table 2 and 3. TSS content was significantly influenced by growing season (Table). In 2013, TSS was significantly higher

compared to the second season (2014). Storage time had a significant effect ($p<0.0001$) on TSS content (Table 2), notably, TSS content increased with storage time. Contrary, storage condition had an insignificant ($p<0.1510$) effect on TSS content. These results are contrary to high TSS content previously reported in low oxygen storage for CA stored 'd'Anjou' pears (Mattheis and Rudell, 2011) and DCA stored 'Cortland' apples (DeLong et al., 2007). The variation in the current study could be due to differences in physiological and biochemical attributes of previously studied cultivars and 'Granny Smith' apples. Moreover, the disruption of DCA for 14 d in RA could also explain this result.

Titratable acidity (TA, expressed as malic acid mg $100mL^{-1}$) was significantly influenced by the season variability ($p=0.0036$; Table 2) and interaction of storage condition and storage time ($p=0.0035$; Table 3). The significant interaction between storage condition and storage time could be attributed to TA loss in RA stored fruit for longer storage times (70 d and 126 d), contrary to higher TA content in repeated DCA stored fruit (Table 3). This result is consistent with previous findings on the influence of low oxygen storage on TA content. DeLong et al. (2007) reported a higher TA content in DCA stored 'Cortland' apples compared to conventional CA after 8 months storage. Studies by Mattheis and Rudell (2011) on the response of 'd'Anjou' pears to low oxygen storage showed a higher TA content at oxygen levels of 0.4 kPa compared to fruit stored in RA. Similarly, DCA stored 'Golden Delicious' apples have been reported to maintain higher TA contents (Gabioud et al., 2009). The higher TA contents in DCA stored fruit demonstrates a reduced respiration process and preserved fruit quality. Therefore intermittent DCA treatments still produce a slightly higher TA for longer stored fruit compared to RA stored fruit.

The ratio of TSS/TA was significantly ($p<0.0002$) influenced by the interaction between storage condition and storage time (Table 3). Seasonal variability and storage time also showed a significant interaction ($p<0.0303$; Table 4). The interaction between storage condition and storage time could be explained by the higher TA loss in RA fruit during storage and higher TA contents in DCA stored fruit in especially longer stored fruit. It could therefore be argued that the lower TA in RA played a major role in keeping TSS/TA higher in this fruit. The TSS/TA ratio is a major contributor to consumer preference and such fruit is associated with better taste (Pathange et al., 2006). Compared to the repeated DCA treatment, RA stored fruit had a higher TSS/TA ratio. Whilst a high TSS/TA ratio improves organoleptic properties (Pathange et al., 2006), fruit should not completely lack acidity, thus

the ability of DCA to retain TA regardless of repeated DCA with an intermittent 14 d in RA storage, is still a highly desired phenomenon.

4.3 Fruit firmness

Fruit firmness was significantly ($p<0.0013$) influenced by the interaction among season, storage condition and storage time (A*B*C). In the 2013 season, fruit firmness in repeated DCA and RA declined by 9% and 43%, respectively from harvest to the 126 d of storage (Fig. 1). However, in 2014 season, firmness declined by 11% and 24% in repeated DCA and RA, respectively. The first season (2013) fruit were harvested slightly firmer and therefore resulted in a higher decrease in firmness from harvest to the last storage and ripening period for RA stored fruit. Repeated DCA therefore still retains the firmness better compared to RA fruit. The difference in firmness retention is consistent with previous uninterrupted DCA storage work that demonstrated high firmness retention in 'Golden Delicious' (Zanella et al., 2008), 'Cortland' apples (DeLong et al., 2007) and 'd'Anjou' pears (Mattheis and Rudell, 2011) stored in DCA compared to either normal air or conventional CA. High fruit firmness retention in DCA storage compared to CA shows the benefit of 'optimised' low oxygen levels during cold storage as previous indicated by Watkins (2008). This benefit is even evident when DCA is done repeatedly with an interruption of RA to source fruit for packing and is still a better option than only RA storage.

4.4 Fruit colour

The market value of apple fruit is associated with ground colour (Zanella, 2003). Postharvest treatments should retard degreening process and maintain ground colour of the fruit. In this present study, an interaction between storage condition and storage time ($p<0.0001$) had a significant effect on ground colour. This interaction could be explained by the rapidly changing ground colour in RA stored fruit from green to yellow with increasing storage time (Fig. 2A). The repeated DCA with an intermittent RA period of 14 d had a positive influence on ground colour retention compared to RA fruit. Unlike the RA stored fruit, ground colour was significantly retained in all DCA treated fruit.

The hue angle (h^0) loss of fruit in the RA storage and the repeated DCA with intermittent RA storage treatment was 3% and 7%, respectively. This finding is similar to when continuous DCA treatments where Zanella (2003) reported a maintained ground colour

in 'Granny Smith' apples exposed to low oxygen levels. Similar results were observed by Mattheis and Rudell (2011) in 'd'Anjou' pears stored in 0.5 kPa O_2 compared to those stored at CA or 1.5 kPa O_2. The influence of changing gas composition during storage exerts a considerable effect on fruit colour and overall fruit quality. It is clear that repeated DCA treatments with an intermittent 14 d at RA has a better effect on green colour retention compared to RA stored fruit. To the best of our knowledge, this is the first study to show that a repeated DCA storage with a 14 d interruption in RA is effective in controlling scald in conditions where scald potential is extremely high.

4.5 Ethylene production

Ethylene production plays a critical role in α-farnesene synthesis and superficial scald development (Gapper et al., 2006; Lurie et al., 2005; Rupasinghe et al., 2000). In fact, ethylene reducing postharvest treatments such as aminoethoxyvinylglycine and 1-MCP retard both α-farnesene and scald incidence (Ju and Curry, 2000; Zanella, 2003). In this study, an interaction between storage condition and storage time had a significant ($p<0.0001$) effect on ethylene production. This interaction could be explained by the markedly higher ethylene production in RA storage contrary to relatively lower ethylene production in the repeated DCA treatment with a 14 d intermittent storage at RA. (Fig. 2B). Our findings are corroborated by those reported by several researchers (Ghahramani et al., 2000; Pesis et al., 2010; Sabban-Amin et al., 2011) who demonstrated that ethylene production is reduced by low oxygen storage conditions. In fact, Whitaker and Solomos (1997) indicated that reduction of scald incidence by low oxygen atmosphere is attributed to low ethylene production. Weber et al. (2015) also reported a lower ethylene production in DCA stored 'Royal Gala' apples in contrast to high ethylene accumulation in CA stored fruit.

There are two major mechanisms of action used by superficial scald inhibitors. The inhibition of α-farnesene oxidation is the major mechanism of DPA (Isidoro and Almeida, 2006; Rudell et al., 2005). On the contrary, 1-MCP reduces the substrate available for oxidation by inhibiting both α-farnesene and ethylene accumulation (Isidoro and Almeida, 2006; Jung and Watkins, 2008). Based on our results, it could be argued that ethylene inhibition is part of the mechanism used by DCA. Apart from its involvement in scald development, ethylene also plays a major role in aroma volatile synthesis (Pechous and Whitaker, 2004). Ethylene production should therefore not be completely eliminated at postharvest. Sabban-Amin et al. (2011) reported a zero ethylene production in 1-MCP treated

fruit whilst a gradual rise in low oxygen treated fruit was found. Ethylene production is associated with *MdACS* and *MdACO* gene (Sabban-Amin et al., 2011). From our results, it could be argued that both *MdACS* and *MdACO* genes were not completely suppressed by repeated DCA with an RA interruption, which may have influenced the ethylene production due to an intermittent O_2 availability needed for ethylene production. Moreover, it could be concluded that in addition to its inhibitory effect on superficial scald, repeated DCA with a 14 d interruption at RA allows 'acceptable' ethylene production for aroma volatile synthesis, an important phenomenon on fruit quality, but was not measured in this study.

4.6 α -Farnesene and MHO production

Scald development is closely associated with α-farnesene accumulation in apple peel. Previous studies have shown that α-farnesene accumulation increases with storage time (Meigh, 1956; Huelin and Kennett, 1958), and its oxidation products such as MHO and CTols induce superficial scald symptoms (Lurie and Watkins, 2012). In this study, an interaction between storage condition and storage time had a significant ($p<0.0001$) effect on α-farnesene production. This interaction could be explained by the markedly lower α-farnesene production in the repeated DCA with intermittent RA stored fruit contrary to RA stored fruit which had a relatively higher production. Regardless of the repeated DCA treatment with an intermittent RA period, fruit had markedly lower α-farnesene accumulation compared to RA stored fruit after 24 to 70 d of storage (Fig. 2C). The effectiveness of low oxygen storage to retard α-farnesene accumulation and scald development has previously been reported. Sabban-Amin et al. (2011) demonstrated a reduced α-farnesene concentration in 'Granny Smith' apples exposed to <0.5% O_2 prior 24 w of cold air storage. The DCA treated fruit resembled the behaviour of initial low oxygen stress and 1-MCP treated fruit by retarding α-farnesene synthesis (Lurie et al., 2005; Pechous and Whitaker, 2004). Our results confirmed the putative role of α-farnesene in scald development.

The inhibition of superficial scald by antioxidant treatments is the evidence of oxidative processes involved in scald development. Thus, superficial scald is closely associated with MHO, an α-farnesene oxidation product. In this study, an interaction between storage condition and storage time also had a significant ($p<0.0001$) effect on MHO accumulation. The difference in MHO accumulation between the storage conditions and an increase with storage time up to 70 d could explain this interaction. MHO accumulation was significantly higher in RA stored fruit (Fig. 2D) after 42 and 70 d of storage, compared to the

repeated DCA stored fruit with an intermittent RA period and consequently resulted to higher scald incidence. This finding was consistent with Sabban-Amin et al. (2011) who observed low MHO accumulation and reduced scald incidence in 'Granny Smith' apples stored in low oxygen storage for 24 months at 0 °C. MHO production and its accumulation in the cuticle is reduced by low oxygen levels either in CA, hypobaric storage or anaerobic treatments (Wang and Dilley, 2000a; Whitaker, 2000). Moreover, Wang and Dilley (2000b) observed a complete MHO suppression after oxygen levels declined from 1.5% to 0.13%. This finding demonstrates that repeated DCA treatments with an intermittent 14 d RA period of 14 d has a marginal effect on α-farnesene oxidation to MHO and that these intermittent RA period during repeated DCA treatments may only become relevant in longer term storage treatments. The decline in MHO content in RA stored fruit after 126 d of storage could suggest a completed role of MHO in scald development. The 2% development of superficial scald in the repeated DCA stored fruit with an intermittent RA period after a total of 126 d of storage coincided with a significantly higher MHO production compared to 70 d of storage. This could be an indication of the negative effect of an intermittent RA period in the repeated DCA treatment on extended fruit storage. Further research is warranted to evaluate this possibility.

4.7 Principal component analysis

To obtain a broad view of metabolomic changes that occurred during storage of 'Granny Smith' apples in DCA or RA, all the biochemical indices measured during the two seasons were subjected to principal component analysis (PCA). The data distribution in the PCA score plot is presented in Fig. 3. The first two principal components (F1 and F2) accounted for more than 92.9% of the total variability. Acceptable explanations could be drawn from the first component (F1) which explains 84.7% of the total variance. The relationships between metabolites were evidenced by short distances between ethylene, α-farnesene and MHO and between TA, hue angle and fruit firmness.

Eigen values and factor loadings of the investigated quality and metabolic parameters showed major differences in RA or the repeated DCA stored fruit with an intermittent RA period (Table 3). The classification of factor loadings is considered strong, moderate and weak when corresponding to loading values of 0.75, 0.75-0.50 and 0.50-0.30, respectively (Liu et al., 2003). The scores can be interpreted by factor loadings (Table 3), with F1 showing strong positive correlations for scald, ethylene, MHO, α-farnesene and TSS, and negative

correlations for TA, ground colour and firmness. In general, this study showed that the repeated DCA stored fruit with an intermittent RA period have a higher acidity, ground colour and firmness; while RA stored fruit have higher scald incidence, ethylene production, α-farnesene and MHO. PCA showed two clusters discriminating the RA and DCA stored fruit. The strong positive correlation and short distance between ethylene production, α-farnesene and MHO showed their biochemical relationship as previously reported (Mir and Beaudry, 1999; Lurie et al., 2005; Pesis et al., 2010; Whitaker, 2004).

5. Conclusion

The results from this trial confirms that the commercially used technique of repeated DCA treatment with an intermittent 14 d RA period is effective in controlling scald up to 70 d of cold storage, especially considering the high scald potential of fruit used in the study. It would therefore be possible, without adversely affecting fruit quality, to source fruit from a DCA cold store and continue the DCA treatment on remaining fruit inside the store should the fruit be stored less than 70 d. The repeated DCA treatment with an intermittent RA period reduced ethylene production, α-farnesene and MHO accumulation. The 2% development of superficial scald after the 126 d of storage could be an indication of the compromised repeated DCA treatment efficacy at an extended storage time. More research is warranted to confirm these results and evaluate the limitations of this technique for further application commercially in fruit where the potential of scald development is high.

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Table 1

Superficial scald incidence in 'Granny Smith' apples stored in refrigerated air (RA) and dynamic controlled atmosphere (DCA) and ripened for 7 d at 20 °C, over two growing seasons.

Season	Storage	Storage Time (days)	Scald (%)
2013	RA	At harvest	0^{f}
		24	0^{f}
		42	67^{d}
		70	99^{a}
		126	100^{a}
	DCA	At harvest	0^{f}
		24	0^{f}
		42	0^{f}
		70	0^{f}
		126	1^{f}
2014	RA	At harvest	0^{f}
		24	0^{f}
		42	51^{e}
		70	72^{c}
		126	95^{ab}
	DCA	At harvest	0^{f}
		24	0^{f}
		42	0^{f}
		70	0^{f}
		126	2^{f}
		Pr>F	
Season (A)			<0.0001
Storage (B)			<0.0001
Storage Time (C)			<0.0001
A*B			<0.0001
A*C			<0.0001
B*C			<0.0001
A*B*C			**<0.0001**

Mean values with different letter (s) in the same column indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test.

Table 2

Changes in TSS and TA in 'Granny Smith' apples cold stored and ripened for 7 d at 20 °C, over two growing seasons.

Season	**TSS (° Brix)**	**TA (mg/100ml)**
2013	11.95a	1.21b
2014	11.78b	1.25a
P-value	**0.0189**	**0.0036**
Storage Time		
At harvest	10.52d	-
24	11.54c	-
42	12.20b	-
70	12.45a	-
126	12.64a	-
P-value	**<0.0001**	-

Mean values with different letter (s) in the same column indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test.

Table 3

Changes in TA and TSS:TA in 'Granny Smith' apples stored in refrigerated air (RA) and dynamic controlled atmosphere (DCA) and ripened for 7 d at 20 °C. Since the season had insignificant effect on the studied parameters, the data for both seasons has been pooled.

Storage Condition	Storage Time (days)	TA	TSS:TA
RA	At harvest	1.39a	7.73g
	24	1.26c	9.39e
	42	1.19df	10.39cd
	70	1.13f	11.21b
	126	1.02g	12.78a
DCA	At harvest	1.39a	7.73g
	24	1.32b	8.43f
	42	1.24cd	9.76de
	70	1.24cde	10.01cde
	126	1.18ef	10.64bc
			Pr>F
	Storage Condition (B)	<0.0001	0.0004
	Storage Time (C)	<0.0001	<0.0001
	B*C	**0.0035**	**0.0002**

Mean values with different letter (s) in the same column indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test.

Table 4

Changes in TSS:TA in 'Granny Smith' apples stored in refrigerated air (RA) and dynamic controlled atmosphere (DCA) and ripened for 7 d at 20 °C, over two growing seasons.

Season	Storage Time	TSS:TA
2013	At harvest	8.06f
	24	8.87e
	42	10.19cd
	70	10.85bc
	126	12.37a
2014	At harvest	7.39g
	24	8.94e
	42	9.97d
	70	10.37bc
	126	11.05b
		Pr>F
	Season (A)	0.0004
	Storage Time (C)	<0.0001
	A*C	**0.0303**

Mean values with different letter (s) in the same column indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test.

Table 5

Factor scores, loadings, eigenvalues and total variance (%) for the first two factors (F1 and F2) based on biochemical and metabolic properties of 'Granny Smith' apples in 2013 and 2014 seasons.

	Factor Scores	
Observation	**F1**	**F2**
2013_At harvest	-3.820	0.848
2013_24d RA	-1.139	-0.168
2013_42d RA	2.038	0.295
2013_70d RA	4.752	0.289
2013_126d RA	5.080	1.755
2013_24d DCA	-2.458	0.061
2013_42d DCA	-0.294	-0.324
2013_70d DCA	0.077	-1.464
2013_126d DCA	1.071	-1.434
2014_At harvest	-4.289	1.002
2014_24d RA	-0.829	-0.326
2014_42d RA	1.691	-0.412
2014_70d RA	-3.820	0.848
2014_126d RA	4.081	0.931
2014_24d DCA	-2.608	0.494
2014_42d DCA	-0.738	-0.592
2014_70d DCA	0.299	-0.836
2014_126d DCA	0.904	-0.967
Loadings		
Ethylene	0.908	-0.083
Scald	0.829	0.469
TSS	0.838	-0.390
TA	-0.867	-0.021
TSS/TA	0.903	-0.057
Texture	-0.828	-0.424
Hue angle (^{0})	-0.853	-0.154
MHO	0.880	-0.182
A-farnesene	0.874	-0.315
Eigenvalue	7.620	0.745
Total variance (%)	84.665	8.273
Cumulative (%)	84.665	92.937

Table 6

This table shows the significance levels of the factors and their interactions of the figures presented below

Source	Significance level Pr>F				
	Ethylene	**Firmness**	**Hue°**	**α-farnesene**	**MHO**
Season (A)	0.4445	<0.0001	0.6370	0.0989	0.2839
Storage (B)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Storage Time (C)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
A*B	0.8255	<0.0001	0.7242	0.2849	0.4526
A*C	0.7805	<0.0001	0.1125	0.8786	0.8698
B*C	**<0.0001**	<0.0001	**<0.0001**	**<0.0001**	**<0.0001**
A*B*C	0.5511	**0.001**3	0.2143	0.9803	0.6797

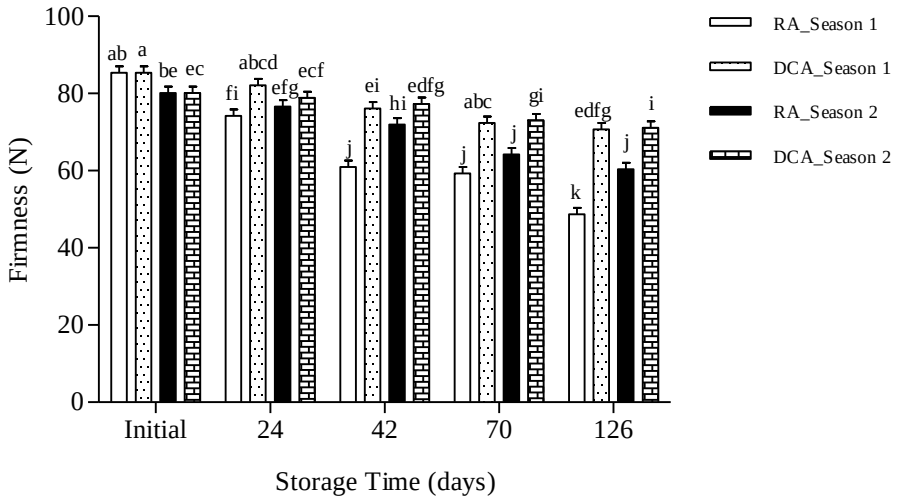

Fig. 1. Effects of the interaction between growing season, storage condition and storage time (p=0.0013) fruit firmness of 'Granny Smith' apples during 126 d of storage at -0.5 °C and additional 6 w of shipment period and 7 d shelf-life. Mean values with different letter (s) indicate significant differences (p<0.05) according to Duncan's multiple range test.

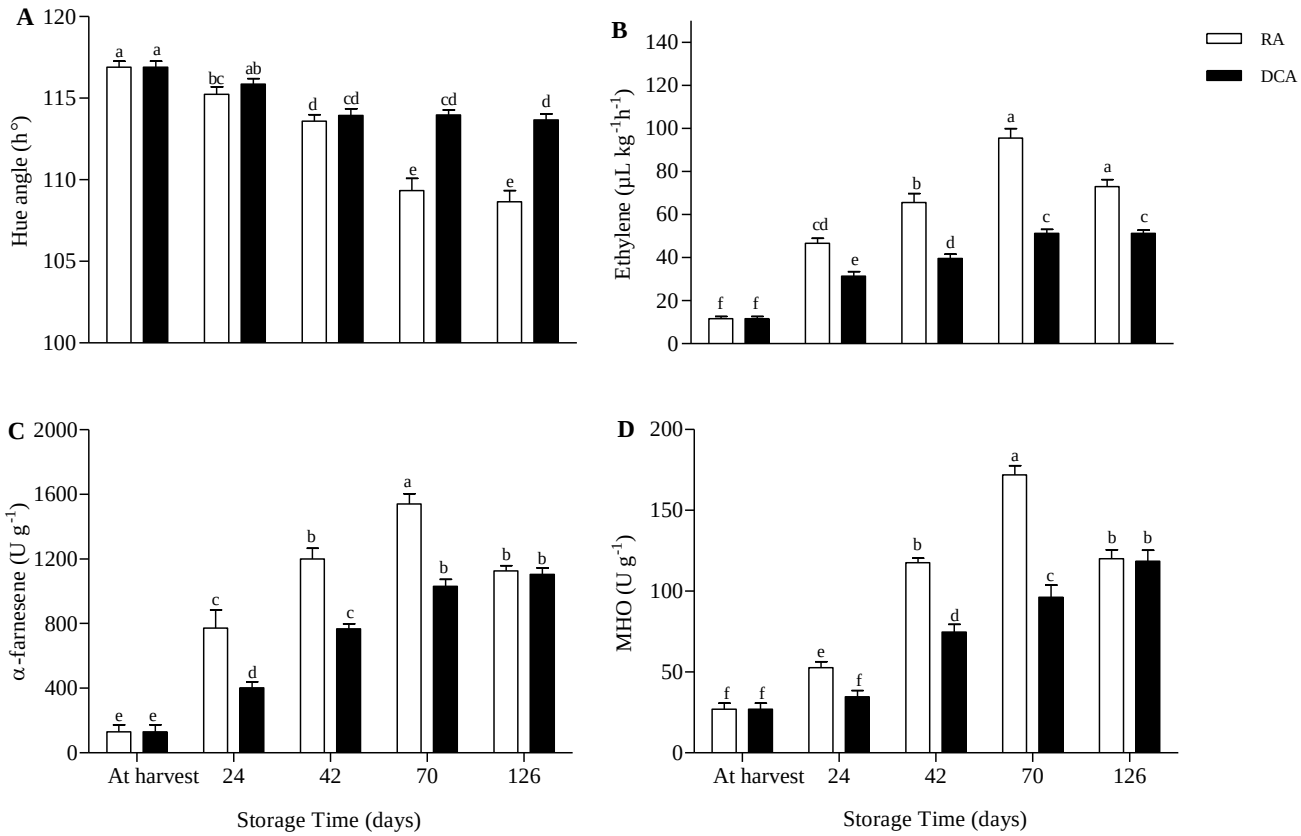

Fig. 2. Effects of the interaction between storage condition and storage time ($p<0.0001$) on hue angle (A), ethylene production (B), and head space accumulation of α-farnesene (C) and MHO (D) of 'Granny Smith' apples during 126 d of storage at -0.5 °C and additional 6 w of shipment period and 7 d shelf-life. Since the season had insignificant effect on the studied parameters, the data for both seasons has been pooled. Mean values with different letter (s) indicate significant differences ($p<0.05$) according to Duncan's multiple range test.

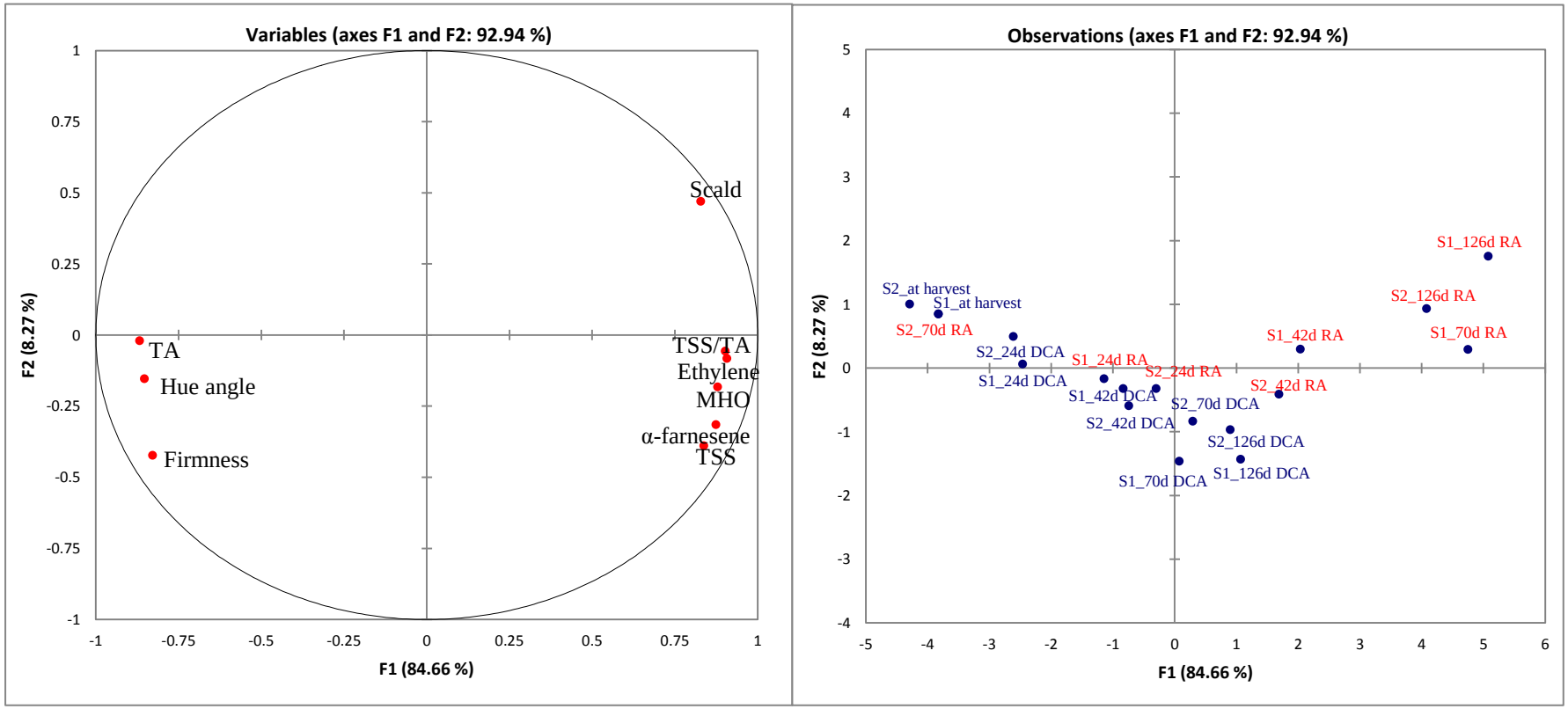

Fig. 3. Variables and observations chart of biochemical and metabolic changes as influenced by RA and DCA storage conditions. Abbreviations: S1-season 1, S2-season 2, RA- refrigerated air, DCA- dynamic controlled atmosphere, TA-titratable acidity, TSS-total soluble solids, MHO-6-methyl-5-hepten-2-one

PAPER 7

Effect of Dynamic Controlled Atmospheres on Volatile Compound Production in 'Granny Smith' apples

Abstract

'Granny Smith' apples were stored in dynamic controlled atmospheres (DCA; 0.3-0.5% O_2/ 1% CO_2) and normal refrigerated air (control) for 12, 16 and 20 w at -0.5 °C. The volatile emitted from the pulp of the fruit was thereafter measured. Total amount of volatiles detected in the control fruit samples were significantly ($p<0.05$) higher than those exposed in DCA. Production of 1-butanol, 1-hexanol and 1-hexen-ol by fruit stored in DCA were 25%, 45% and 27%, respectively, of the amounts detected in the control. DCA treatments also resulted in higher emission of ethanol compared with fruit stored in RA. Production of 2-methylpropyl acetate by DCA stored fruit was 19% of that produced by the control fruit. Aldehydes emissions were lower in DCA compared to control fruit. Esters were the main volatiles detected (50%). The highest emission of ester volatiles was obtained from the control fruit. The known characteristic flavour volatiles in apples, ethyl-2-methylbutyrate, ethyl hexanoate and hexyl acetate were significantly high in this study. Their contribution was higher throughout the storage duration. The TA loss was significantly reduced by DCA treatments compared to normal refrigerated air storage.

Keywords: *Malus domestica*, dynamic controlled atmosphere, volatiles, esters, alcohols, aldehydes, fruit quality

1. Introduction

Dynamic controlled atmosphere (DCA) storage technology is becoming more popular in commercial storage of apples for reducing postharvest physiological disorders such as superficial scald and retaining fruit quality during long term storage. Previous studies on controlled atmosphere (CA) and low oxygen storage have reported a suppressed production of aroma compounds, a main contributor to apple flavour (Fellman et al., 2000; Mattheis et al., 2005). Ethylene plays a critical role in the synthesis of aroma compounds (Pechous and Whitaker, 2004). In fact, postharvest storage methods should not completely suppress ethylene production (Dandekar et al., 2004). However, low oxygen storages such as CA, DCA, and ultra low oxygen (ULO) are known for retarding ethylene production (Ghahramani

et al., 2000; Pesis et al, 2010; Sabban-Amin et al., 2011). Interestingly, Whitaker and Solomos (1997) reported that reduction of scald incidence by low oxygen atmospheres is attributed to low ethylene production in stored fruit. Reduced ethylene production in DCA stored 'Gala' apples in contrast to high ethylene accumulation in normal refrigerated air has been reported (Mattheis et al., 1998).

There are an array of factors influencing the production of volatile compounds and flavour development in apples. For instance, fruit maturity is an important factor influencing both the volatile and flavour development in 'Delicious' (Fellman et al., 2003) and 'Fuji' apples (Echeverria et al., 2004). Additionally, postharvest treatments and storage conditions have a significant effect on evolution of volatiles and their profile (Lurie et al., 2002; Mattheis et al., 2005). An array of chemical methods have been used to control postharvest physiological disorders. However, increasing health concerns have necessitated the use of nonchemical methods such as CA and ULO. On the other hand, these technologies do not completely control superficial disorder. An alternative to chemical treatments, DCA, has been shown to effectively preserve apple quality (Zanella et al., 2005; DeLong et al., 2007). This technology has gained interest particularly to organic markets which uses only approved chemical treatments on food products.

DCA has been shown to preserve fruit quality parameters such as firmness in 'Gala', 'Golden Delicious' and 'Cortland' apples (Mattheis et al. 1998; Zanella et al., 2008; DeLong et al., 2007), colour in 'Granny Smith' apples and 'd'Anjou' pears (Zanella, 2003; Mattheis and Rudell, 2011), total soluble solids and titratable acidity in 'Cortland' and 'Golden Delicious' apples (DeLong et al., 2007; Gabioud et al., 2009). There is limited information on the influence of DCA on aroma compounds. Mattheis et al. (1998) reported no negative effects of DCA on volatile compounds in 'Gala' apples. DCA storage technology has also been reported to preserve aroma compounds in 'Pinova' apples (Raffo et al., 2009). No information about the effect of DCA on volatile compounds in 'Granny Smith' apples has been reported to date. In addition to fruit maturity and seasonal variability (Fellman et al., 2003; Echeverria et al., 2004), the influence of storage treatment on volatile production differs amongst cultivars. The objective of this research study was to compare the volatile profile of 'Granny Smith' apples after three, four and five months of DCA storage with that stored in normal refrigerated air.

2. Materials and methods

2.1 Fruit source, treatments and storage

Apple fruit (*Malus* x *domestic* Borkh.) cv. 'Granny Smith' grown in a commercial orchard were hand-picked from Valley Green Farm in Grabouw, South Africa (34^0 12'12" S, 19^0 02'35" E) at optimal maturity (172 DAFB). Uniformly sized fruit with diameter of 70±2 mm and mass of 160±5 g were randomized to provide 3 replications of 60 fruit each and stored in air at -0.5 °C and 95% relative humidity for three to five months. Another batch of fruit was stored at DCA storage under the same conditions as above. Each DCA storage chamber had a sensor in a plastic basket with a sample of 6 apples. DCA sets points were determined by a chlorophyll fluorescence non-destructive monitoring system (HarvestWatch, Satlantic Inc, Halifax, Canada) which can predict and indicate the occurrence of low oxygen stress (Prange et al., 2003; Wright et al., 2012). In this study, the DCA was established within 48 h after harvest, using compressed air and CO_2 plus N_2 from a membrane generator (Isosep, Isolcell, Italy). The gas composition of the storage chamber were analysed at 90 minutes intervals and adjusted when necessary. The O_2 levels generally ranged between 0.3 to 0.5% whilst CO_2 was maintained at 1% at 95% RH.

2.2 Titratible acidity

Fruit was juiced using a LiquaFresh juice extractor (Mellerware, South Africa) to measure titratible acidity (TA). Titratible acidity expressed as malic acid, was measured from ten fruit composite sample (3 replicates per treatment) at room temperature using titration to an endpoint of pH 8.2 (NaOH), a Metrohm 862 compact titrosampler was used (Herisau, Switzerland).

2.3 Extraction procedure of volatile compounds and chromatographic analyses

Fruit was homogenized according to the method reported by Lurie et al. (2002). Briefly, 200 g (three replicates per treatment) of apple pulp (slices cut from ten fruits per replicate, excluding peel and seeds) was homogenized with LiquaFresh blender (Mellerware, South Africa) for 2 min. Approximately 10 mL of apple juice was placed in 20 mL SPME vials with PTFE screw cap and 50 µL of the internal standard solution (3-octanol, 1 µg mL^{-1}

in methanol). To enhance the extraction efficiency of volatiles from the solution to the headspace, 1 mL of NaCl at 30% (m/v) was added.

The volatiles were trapped and extracted from the vials onto the fibre from the headspace (Mayuoni-Kirshinbaum et al., 2012; Caleb et al., 2013). The vials were equilibrated for 10 min at 50 °C in the CTC autosampler incubator. After equilibration, a 50/30 µm divinylbenzene/-carboxen/-polydimethylsiloxane coated fibre was then exposed to the sample headspace for 20 min at 50 °C. After extraction, the trapped volatile compounds from the fibre coating were desorbed for 2 min in the injection port of the gas chromatograph operated in a splitless mode. The temperature was maintained at 250 ºC for the injection. The fibre was cleaned after each sample heating for 10 min in the fibre conditioned station maintained at 270ºC.

Chromatographic separation of the extracted volatiles was performed on a Agilent 6890N (Agilent, Palo Alto, CA) connected through a transfer line to a Agilent 5975B MS (Agilent, Palo Alto, CA) mass spectrometer detector. The GC–MS system was equipped with a polar DB-FFAP column from J&W (part number 122-3263) with the following dimensions: (60 m length; 250 µm internal diameter; and 0.5 µm film thickness). Helium was used as carrier gas at a flow rate of 1.3 mL min^{-1}. The oven temperature program was as follows: initial temperature of 40 °C for 5 min; then ramped at 5 °C min^{-1} up to a final temperature of 230 °C with a final hold time of 6 min. The ion source and quadropole were maintained at 240 °C and 150 °C, respectively. The transfer line temperature was maintained at 280 °C. Volatile compounds were tentatively identified by comparing their mass spectra with the spectral libraries (NIST 95, version 2.0) with comparison quality of >90%. The relative proportion (%) of each volatile compound was calculated as a percentage ratio of peak area of each compound to the total peak area of all identified compounds (Fawole and Opara, 2013).

3. Statistical analysis

Data was subjected to Statistica 11 (StatSoft Inc. Oklahoma, USA) for analysis of variance (ANOVA) according to Duncan's multiple range test. Graphical data presentations were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc. San Diego, USA).

4. Results and discussion

Dynamic controlled atmospheres completely prevented the development of superficial scald during the normal refrigerated storage and the subsequent holding at 20 °C. In contrast, the control fruit had 85 to 100% scald incidence after the storage period (data not shown). The loss of TA was significantly ($p<0.05$) higher in the control than that of fruit from DCA storage (Table 1). Titratable acidity loss increased with storage time (Table 2). These findings were in agreement with Mattheis et al. (1998) who found reduced TA loss in DCA stored 'Gala' apples. This result has further shown the benefits of low oxygen storage and their potential in enhancing the aroma of 'Granny Smith' apples.

The GC-MS analysis of the volatile compounds from the 'Granny Smith' apples pulp allowed the identification of 10 esters, 2 aldehydes, 6 alcohols, 1 sesquiterpenes and 1 ketone (Table 1 and Fig. 2). The production of volatile compounds was strongly influenced by storage conditions examined in our experiment. Total amount of detected volatiles in the fruit after storage in DCA was significantly ($p<0.05$) lower than the control (Table 1). A significant interaction between storage time and storage condition ($p<0.0001$) had an effect on total volatile levels. Total volatile level appeared to be higher in the control fruit when compared to DCA during the whole storage duration (Fig. 3). This finding corroborates with Raffo et al. (2009) who reported a higher total level of detected volatiles in normal air compared to DCA in 'Pinova' apples stored for 4 months. In this study, this observation was largely influenced by ester production with ethyl hexanoate, hexyl acetate and ethyl 2-methylbutyrate being the most abundant esters detected (Table 1). In the control treatment, hexyl acetate amount was on average 2-fold more than that of DCA (Table 1; Fig. 1). This trend was also reported by Mattheis et al. (1998), Raffo et al. (2009) and Echeverria et al. (2004) in 'Gala', 'Pinova' and 'Fuji' apples, respectively, after CA or ULO storage plus ripening period. Ethyl acetate emissions were not significantly influenced by storage conditions; however, the control fruit had relatively higher emissions. Similarly, Fellman et al. (1993) found ethyl acetate concentrations to increase with oxygen levels in 'Rome' apples stored for 9 months at 0 °C. Another investigation in 'Granny Smith' apples showed ethyl acetate to be severely decreased in low oxygen (0.13% O_2) storage conditions (Wang and Dilley 2000).

Methyl butanoate amounts from the control fruit were significantly higher compared to DCA fruit. In fact, this volatile compound was almost non-existant in DCA. Similarly, the

amount of other ester volatiles were relatively lower in DCA storage when compared to the control fruit. For instance, propyl butyrate emissions from DCA stored fruit were 10% of amounts detected from the control fruit. Storing fruit in DCA led to a significantly ($p<0.05$) reduction of butyl butyrate and methyl hexanoate. Butyl butyrate emissions in DCA were 6%, of the emissions in the control fruit; however, methyl hexanoate was not detected throughout the examined storage duration. The inhibitory effect of low oxygen levels on butyl butyrate amount has previously been reported (Mattheis et al., 1998).

Ethyl hexanoate is one of the ester compounds contributing to the overall aroma of fruit (López et al., 1998). The production of ethyl hexanoate was significantly influenced by the storage condition (Table 1). On average, fruit stored in DCA produced 49% less ethyl hexanoate in comparison to the control fruit. This result is contrary to Mattheis et al. (1998) who found insignificant influence of either normal refrigerated air or CA on ethyl hexanoate emissions. Interestingly, Mir and Beaudry (1999) on their study on the influence of DPA on volatile compounds of 'Cortland' apples also found no significant difference on treated or untreated fruit. Based on these findings, it could be argued that DCA has a limited effect on ethyl hexanoate production. This notion could be supported by López et al. (1998) who found ethyl hexanoate emissions to be significantly lower in CA (1% O_2/1% CO_2) stored of 'Starking Delicious' apples compared to refrigerated air.

Storage conditions significantly influenced the emission of 2-methylpropyl acetate. Fruit stored in DCA had 19% of the amounts detected from the control fruit after storage. These results are similar to those shown by López et al. (1998) in 'Starking Delicious' apples after 7 months storage in CA. Ethyl 2-methylbutyrate was high in DCA stored fruit compared to the control fruit. This finding corroborates with López et al. (1998) who found that low oxygen storage (1% O_2/1% CO_2) increase ethyl-2methylbutyrate emission in 'Starking Delicious' apples stored for 5 months at 1 °C. In contrast, another investigation on 'Golden Delicious' apples showed that ethyl 2-methylbutyrate levels decrease with oxygen levels (López et al., 2000). This difference in cultivars response could be explained by the availability of substrate responsible for ethyl-2-methylbutyrate production. Contrary to 'Golden Delicious' apples, it could be argued that the substrate responsible for ethyl-2-methylbutyrate production is more active in low oxygen conditions for cultivars such as 'Granny Smith' and 'Starking Delicious' apples. Storage conditions had a significant effect on hexyl butyrate production. Amounts of hexyl butyrate emitted by the control fruit were

higher than the amounts detected in DCA throughout the storage duration. López et al. (1998) observed similar findings in 'Starking Delicious' apples, fruit stored in CA had lower hexyl butyrate concentrations compared to fruit stored in normal refrigerated air.

An interaction between storage time and storage condition ($p=0.0041$) had a significant effect on total amount of esters. This interaction was possibled influenced by DCA which appeared to have a lower amount of esters (Fig. 4). A similar trend has previously been reported in 'Gala' apples stored in normal refrigerated air or static CA or DCA (Mattheis et al., 1998). The same study demonstrated that ester emissions increase with oxygen concentration. Fellman et al. (2000) reported that biosynthesis of certain volatile compounds highly depends on oxygen levels during storage. The reduced ester accumulation in DCA could be linked to reduced biochemical oxidative processes in this fruit. Esters are identified as the major contributors to flavour of different apples cultivars (Mattheis et al. 1998; Mir and Beaudry, 1999; Echeverría et al., 2004; Raffo et al., 2009). The low ester production in DCA as found in this study could influence organoleptic properties of this fruit. Previous studies have shown that the negative effects of CA on volatile compounds depend on both storage time and atmospheres. For instance, López et al. (2000) reported a significant flavour loss in CA (30 L m^{-3} O_2/30 L m^{-3} CO_2) stored 'Golden Delicious' apples which led to lower consumer acceptance, in the same study, CA with relatively lower oxygen level (20 L m^{-3} O_2/20 L m^{-3} CO_2) performed better and resulted in good quality fruit with higher consumer acceptance. Similarly, Fellman et al. (2000) found that the flavour of 'Delicious' apples is reduced by prolonged lower O_2 and high CO_2 storage. The constantly changing storage atmospheres in DCA depending on the response of fruit to anaerobic stress could be playing a critical role in preserving the flavour in 'Granny Smith' apples. Moreover, in spite of low total amount of esters in DCA, reduced loss of TA together with high levels of ethyl 2-methylbutyrate, hexyl acetate and ethyl hexanoate significantly contribute to apples flavour (Mattheis et al., 1998; Echeverria et al. 2004; Fellman et al., 2000; Lara et al., 2006). Young et al. (1996) indicated that these volatile compounds contribution to a fresh-green and fruit odors. These findings are in agreement with Raffo et al. (2009) who speculated that DCA has the potential to preserve flavour of 'Pinova' apples. Future research should focus on correlating analytical and sensory measurements of DCA stored 'Granny Smith' apples.

Six alcohols comprising of 1-butanol, 1-hexanol, 1-pentanol, 2-hexen-1-ol, ethanol and 6-methyl-5-hepten-2-ol were detected in this study. Except for ethanol, the influence of

DCA on alcohol production was analogous to ester production. There was a marked reduction of alcohol levels in DCA compared to fruit stored in normal air (Table 1). On average, 1-butanol, 1-hexanol and 1-hexen-ol emissions in DCA stored fruit were 25%, 45% and 27% of the amounts detected in the control fruit after storage. This result confirmed the previous observation on 'Pinova' apples (Raffo et al., 2009), indicating that both 1-butanol and 1-hexanol emissions are reduced by DCA storage. Moreover, López et al. (1998) also reported low alcohol emissions (including 1-butanol, 1-hexanol, 2-methyl-1-butanol) in 'Starking Delicious' apples stored in CA (1% O_2/1% CO_2) for 7 months. Although storage duration had insignificant effect on 1-pentanol production, this alcohol was not detected in DCA stored fruit. This finding was contrary to López et al. (1998) who reported 1-pentanol emissions in CA stored 'Starking Delicious' apples. Previous research has shown that alcohols are influenced by storage conditions (López et al., 1998); moreover, alcohols play a critical role synthesis of flavour-associated esters. For instance, Harb et al. (1994) and Altisent et al. (2008) indicated that 1-hexanol favours the production of hexyl acetate. Interestingly, in this present study, emissions of hexyl acetate in the control fruit paralleled high emissions of its precursor (1-hexanol) which was the second most predominant alcohol after ethanol. A similar trend has previous been reported in 'Fuji' apples stored in CA or ULO for 7 months (Lara et al., 2006; Altisent et al., 2008). The control fruit were characterized by higher emission of butyl butyrate probably as a result of higher availability of 1-butanol, its precursor. An investigation by Lara et al. (2006) on 'Fuji' apples also reported a similar trend.

A quite different picture was shown by ethanol production (Fig. 2). An interaction between storage time and storage condition ($p<0.0001$) had a significant effect on ethanol production. Ethanol levels were clearly high in DCA stored fruit compared to the control fruit. This finding shows that metabolic activity favouring ethanol accumulation increases in low oxygen storages (Mattheis et al., 1991). Previous studies on 'Delicious' apples have shown that ethanol accumulation during low oxygen storage triggers the synthesis of ethanol-derived esters (Mattheis et al., 1991). The higher emission of ethyl 2-methyl butanoate in low oxygen stored fruit is often linked to higher availability of ethanol, its precursor (Lara et al., 2006). Ethanol-derived esters generally have low organoleptic threshold (Flath et al., 1967) and these volatiles often produce off-flavours. In this present study, there was only one ethanol derived ester was detected. It is doubtful if the high ethanol production in DCA stored fruit could negatively impact organoleptic properties of 'Granny Smith' apples. We therefore

want to argue that the high ethanol accumulation in DCA stored fruit is part of the mechanism that DCA uses to inhibit superficial scald and prolong fruit quality. This concept has previously been proposed by Ghahramani and Scott (1998) who indicated that low O_2 storages control superficial scald partly by increasing endogenous ethanol production. Wang and Dilley (2000) later hypothesized that exposing fruit to initial low O_2 stress before CA storage induces ethanol synthesis and consequently leads to reduced α-farnesene and MHO accumulation. In their study on the efficacy of initial low O_2 stress to control superficial in 'Granny Smith' apples, Wang and Dilley (2000) found ethanol accumulation to be directly related to inhibiting MHO and reducing scald incidence. Interestingly, both α-farnesene and MHO were lower in DCA stored fruit even though they were not significantly different from the control fruit. Based on these findings, it could be argued that, to a certain extent, anaerobic metabolites are highly beneficial for postharvest fruit quality (Pesis, 2005).

Aldehydes emissions were influenced by storage conditions. Fruit stored in DCA had significantly lower hexanal and tr-2-hexenal emissions compared to the control treatment. This finding could be compared with Raffo et al. (2009) who reported a strong relationship between the suppression of alcohols and accumulation of aldehydes in DCA stored 'Pinova' apples. Based on these findings, it could be argued that DCA reduces the oxidation of aldehydes to alcohol (Brackmann et al., 1993; Raffo et al., 2009). In another investigation, aldehydes accumulation was lower in CA stored 'Delicious' apples (Mattheis et al., 1991). Like other volatiles, the evolution of aldehydes could also be cultivar dependent.

5. Conclusion

DCA had an inhibiting effect on total volatile emissions. Total ester production was reduced in DCA stored fruit throughout the storage duration. The substantial amounts of ethyl 2-methylbutyrate, ethyl hexanoate and hexyl acetate production shows that DCA is not only effective in controlling the incidence of superficial scald on 'Granny Smith' apples, fruit flavour is also better preserved. The emitted ethanol in DCA is probably involved in mechanism of action for controlling superficial scald in 'Granny Smith' apples. Further research on correlating analytical and sensory measurements of DCA stored 'Granny Smith' apples is warranted.

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Table 1

Titratable acidity and volatile compounds (peak area %) emitted from the pulp of 'Granny Smith' apples stored in refrigerated air (control) or DCA. Mean values with different letters in the same row indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test. ND-not detected. Statistical differences are therefore per compound as influenced by the storage condition and does not compare compounds to each other.

Compound	Control	DCA
Esters		
Ethyl acetate	1.29a	0.17b
Methyl butanoate	1.19a	0.05b
Hexyl acetate	7.83a	1.70b
Propyl butyrate	0.61a	0.06b
Butyl butyrate	0.32a	0.02b
Methyl hexanoate	1.40a	ND
Ethyl hexanoate	5.09a	2.54b
2-methylpropyl acetate	1.66a	0.32b
Ethyl 2-methylbutyrate	1.53b	6.45a
Hexyl butyrate	1.39a	0.27b
Alcohols		
1-Butanol	1.94a	0.49b
1-Hexanol	4.81a	2.16b
1-Pentanol	0.66a	ND
2-Hexen-1-ol	2.65a	0.72b
6-Methyl-5-hepten-2-ol	2.78a	1.12a
Aldehydes		
Hexanal	4.87a	1.04b
Tr-2-Hexenal	3.43a	1.83b
Ketones		
3-Octanone	3.68a	2.88a
Sesquiterpenes		
Alpha. farnesene	3.66a	1.56a
Titratable acidity	0.67b	1.12b

Table 2

The effect of storage time on 1-butanol, 2-methylpropyl acetate (peak area %) and titratable acidity of 'Granny Smith' apples. Mean values with different letters in the same column indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test. ND-not detected. Statistical differences are therefore per compound as influenced by the storage condition and does not compare compounds to each other.

Storage Time (weeks)	1-butanol	2-methylpropyl acetate	Titratable acidity (mg/100ml)
12	0.95b	0.90a	1.21a
16	1.75a	1.25a	0.75b
20	0.94b	ND	0.72b
P-value	**0.0241**	**0.0137**	**0.0048**

Table 3

This table shows the significance levels of the factors and their interactions of the figures presented below

Source of variance	Hexyl acetate	Ethanol	Total volatiles	Total esters
Storage Time (A)	0.0016	<0.0001	0.0584	0.0132
Storage Condition (B)	<0.0001	<0.0001	0.0041	<0.0001
A*B	**<0.0001**	**<0.0001**	**0.0531**	**0.0041**

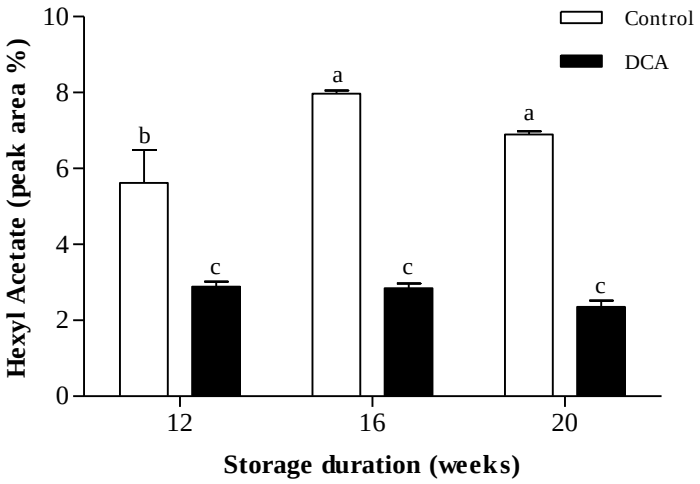

Fig. 1. Accumulation of hexyl acetate (%) of 'Granny Smith' apples following normal air or DCA storage. Bars with different letters indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test.

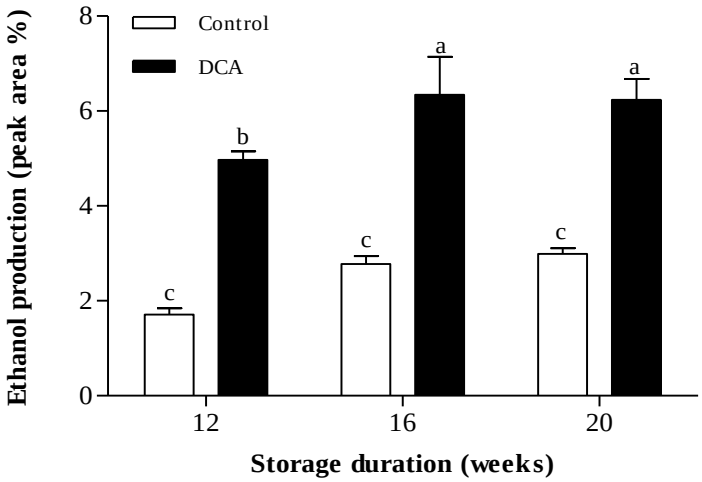

Fig. 2. Ethanol production of 'Granny Smith' apples following normal air or DCA storage. Bars with different letters indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test.

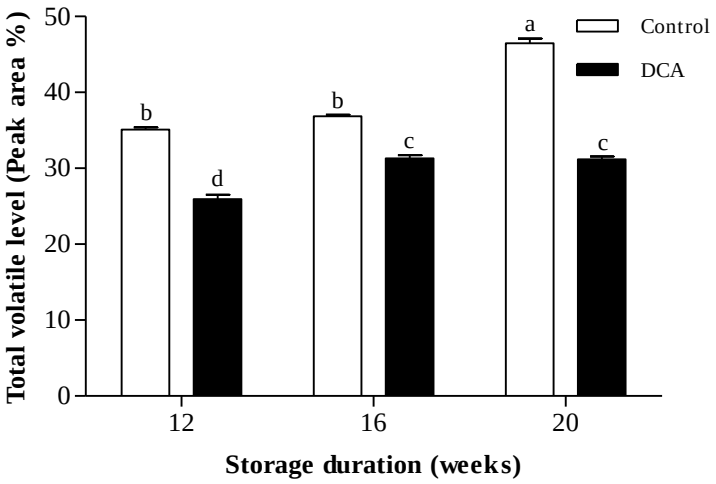

Fig. 3. Total volatile production of 'Granny Smith' apples following normal air or DCA storage. Bars with different letters indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test.

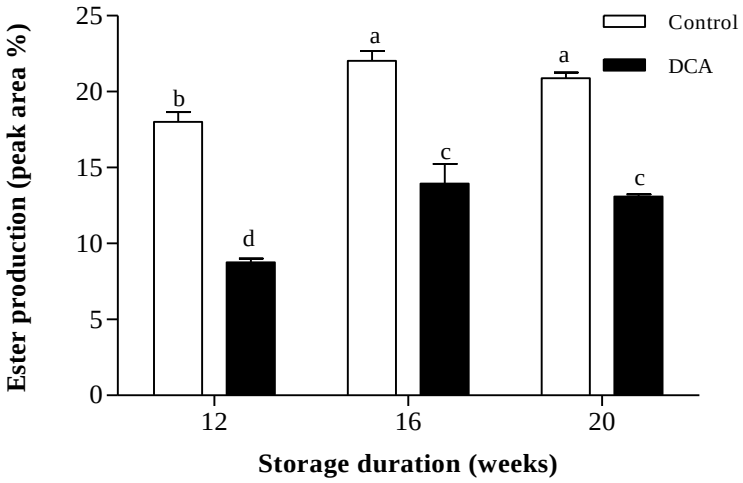

Fig. 4. Ester production of 'Granny Smith' apples following normal air or DCA storage. Bars with different letters indicate statistically significant differences ($p<0.05$) according to Duncan's multiple range test.

PAPER 8

Identification of Effective Predictive Variables for Superficial Scald Development in 'Granny Smith' Apples

Abstract

'Granny Smith' apples harvested at different times and stored in normal air were used to identify variables that could be used to predict superficial scald. The changes in scald-associated volatile compounds such as ethylene, α-farnesene and 6-methyl-5-hepten-2-one (MHO), antioxidants capacity, ascorbic acid concentration (AsA) and lipid peroxidation were monitored over storage time. The result showed that superficial scald is positively correlated with MHO accumulation, ethylene production and lipid peroxidation. Fruit antioxidant capacity and the concentration of ascorbic acid were negatively and strongly correlated to scald development, respectively. It was shown that α-farnesene concentrations were not always positively correlated with scald development. After two years of study, it was shown that the relationship between scald development and MHO fitted the quadratic equation (model A): $y = -0.01[MHO]^2 + 1.69[MHO] - 29.08$ ($R^2 = 0.98$). The results also indicated ethylene and lipid peroxidation as potential predictors of scald development (model B): $y = 0.67$ [Ethylene] + 8.67 [MDA] ($R^2 = 0.94$). Stepwise multiple regression indicated that the best combination of predictor variables for scald development were MHO, antioxidant capacity (FRAP), ascorbic acid concentration and lipid peroxidation (model C): $y = 0.51$ [MHO] – 0.93 [FRAP] + 2.90 [AsA] + 9.82 [MDA] ($R^2 = 0.94$). After validation, model C proved to be the most reliable predictor for scald development, but has limited practical application in industry at this stage due to the extensive wet chemistry needed to quantify the inputs variables. Although model A which only focuses on MHO dynamics during storage was not the most reliable predictor of scald, it would be a handy and easy predictor from a practical viewpoint.

Keywords: superficial scald, α-farnesene, 6-methyl-5-hepten-2-one, ascorbic acid, antioxidant capacity

1. Introduction

Superficial scald is a common postharvest physiological disorder in particular 'Granny Smith' apples. If not controlled, it results to postharvest losses and thereby reducing net income for the producers. Different postharvest techniques including chemical treatments are used to combat apple scald. Therefore, if a prediction model for scald susceptibility could be developed, the use of chemical treatments such as diphenylamine (DPA) and 1-methlycyclopropene (1-MCP) could be reduced (Lourens and Malherbe, 1999). Moreover, such a prediction model could be very instrumental in planning postharvest chain. Previous prediction models have used both preharvest and postharvest variables. For instance, Thomai et al. (1998) used temperatures before harvest as the prediction variable for superficial scald in apples. In their study, fruit exposed to less than 10 °C for 120 to160 h before harvest was correctly predicted to be highly resistant to scald. The levels of total lipids, waxes and fatty acids on the peel increased with time of exposure to 10 °C. Furthermore, high unsaturated fatty acid content, a key phytochemical for retarding membrane damage, was recorded on this fruit. Diamontidis et al. (2002) also reported high unsaturated fatty acids concentration with increasing exposure of fruit to preharvest temperatures below 10 °C. Unsaturation of fatty acids in conditions that induce scald resistance has previously been recorded. For instance, Thomai et al. (1998) and Diamontidis et al. (2002) reported high fatty acid unsaturation index in fruit exposed to less than 10 °C for more than 100 h before harvest. Although low preharvest temperatures aided scald prediction, Goudkovski et al. (1994) proved that maximum preharvest temperatures are integral in developing preharvest-based scald prediction models.

Recent research efforts have focused on utilization of postharvest biomarkers to predict scald. The ratio of α-farnesene to CTols has showed to be a potential predictor for scald development (Zhang and Shu, 2003). However, high CTols regardless of low scald incidence in late harvested fruit reduces the precision of this model. Recently, a scald model based on the rate of conjugated trienes accumulation (Giné Bordonaba et al., 2013) has been developed and validated. This model has been described as sensitive and having good prediction power. However, different climatic and storage conditions or technologies influence the behaviour of a prediction model. The most recent attempts to predict superficial scald is the use of "index of absorbance difference" (IAD), as measured with a DA-Meter, a portable device based on NIR, to check fruit maturity and predict scald development (Farneti

et al., 2015). The ability of IAD has shown that non-destructive techniques for quantifying scald-associated metabolites should be the epitome of scald research in the next decade. This rapid and non-destructive method showed scald incidence to be closely correlated to ripening stage, generally, immature fruit showed higher scald development. Farneti et al. (2015) further indicated that IAD could be integrated into pack-line to optimise postharvest management.

MHO is another α-farnesene oxidation product that is strongly associated with scald development (Wang and Dilley, 2000a); however, no scald prediction model has made attempts to use it as a predictive biomarker for scald development. In chapter 3 and 4, multiple factors were shown to be strongly associated with certain metabolites. Therefore, the objective of this preliminary study is to identify variables with good predictive power that could be used to predict superficial scald development of 'Granny Smith' apples in South Africa. The identified variables could later be used to develop a prediction model; moreover, such variables could be instrumental in developing non-destructive techniques for quantifying scald-associated metabolites in future. Due to the nature of the study, only postharvest biochemical parameters were considered for the development of the prediction models.

2. Materials and methods

2.1 Fruit source and treatments

The study was performed on pre-optimally and optimally harvested 'Granny Smith' apples during 2012/2013 and 2013/2014 growing seasons. Fruit free from visible external damage and blemishes were hand-picked from Valley Green Farm in Grabouw (34^0 12'12" S, 19^0 02'35" E) at 165 and 172 d after full bloom (DAFB) (which are commonly considered in the fruit industry as pre-optimal and optimal maturity periods, respectively). Uniformly sized fruit with diameter of 70±2 mm and mass of 160±5 g were randomly divided into 3 replications of 100 fruit each. Fruit was stored for 16 w in normal refrigerated air (-0.5 °C, 95% RH). Normal air was used to induce the highest incidence of superficial scald. At 7 d after storage at ambient conditions (20 °C and 65% RH), scald incidence was recorded as the percentage of fruit with superficial scald symptoms. Scald associated variables were also measured as detailed below.

2.2 Ethylene production and headspace volatile analysis

Ethylene was measured as described by Öz and Ergun (2009) with slight modifications. Briefly, six fruit per replicate were used to measure ethylene production. Each fruit was weighed using an electronic balance (Mettler Toledo, Switzerland) with an accuracy of 0.01g, and thereafter enclosed in 1 L airtight glass jar with a rubber septum for 1 h at 20 °C. An infrared ethylene analyser (ICA56 ppm, United Kingdom) was used for measurements and the results were expressed as $\mu L C_2H_2 kg^{-1} h^{-1}$.

Fresh peel (5 g) was weighed into a 20 mL solid phase microextraction (SMPE) glass vials. 10 µL of 3-octanol internal standard was added, and the vials were sealed. Vial headspace was analysed according to Mayuoni-Kirshinbaum et al. (2012) and Caleb et al. (2013). The vials were equilibrated for 10 min at 50 °C in the CTC autosampler incubator. After equilibration, a 50/30 µm divinylbenzene/-carboxen/-polydimethylsiloxane coated fibre was then exposed to the sample headspace for 20 min at 50 °C. After extraction, the trapped volatile compounds from the fibre coating were desorbed for 2 min in the injection port of the gas chromatograph operated in a splitless mode. The temperature was maintained at 250 ºC for the injection. The fibre was cleaned after each sample heating for 10 min in the fibre conditioned station maintained at 270 ºC. Chromatographic separation of the extracted volatiles was performed on a Agilent 6890N (Agilent, Palo Alto, CA) connected through a transfer line to a Agilent 5975B MS (Agilent, Palo Alto, CA) mass spectrometer detector. The GC–MS system was equipped with a polar DB-FFAP column from J&W (part number 122-3263) with the following dimensions: (60 m length; 250 µm internal diameter; and 0.5 µm film thickness). Helium was used as carrier gas at a flow rate of 1.3 mL min^{-1}. The oven temperature program was as follows: initial temperature of 40 °C for 5 min; then ramped at 5 °C min^{-1} up to a final temperature of 230 °C with a final hold time of 6 min. The ion source and quadropole were maintained at 240 °C and 150 °C, respectively. The transfer line temperature was maintained at 280 °C. Alpha-farnesene and MHO were identified by a library search and quantified using abundance characteristic ion 93 and 108, respectively. Generally, α-farnesene gave a single peak at 22.2 min while MHO gave a peak at 16.6 min. A reading of 10^4 in abundance was defined as one unit and expressed as U g^{-1} (Ju and Curry, 2000).

2.3 Ascorbic acid content

Ascorbic acid content was quantified according to Fawole et al. (2012) with slight modifications. The mixture was vortexed for 30 s before being ice-sonicated for 3 min, and thereafter centrifuged at 17764 g for 15 min at 4 ºC. In triplicates, the extract (1 mL) was mixed with 9 mL of 2, 6-dichlorophenolindophenol dye (0.0025%). To ensure that only ascorbic acid is measured, the absorbance of the mixture was read at 515 nm within 30 min of incubation in dark environment (Barros et al., 2007). Ascorbic acid content was calculated using the calibration curve of authentic $_{L}$-ascorbic acid (0.01–0.1 mg mL^{-1}), and the results were expressed as ascorbic acid equivalent (AAE) per grams dry matter (mg AAE mg g^{-1}DM).

2.4 Determination of total phenolics

Total phenolic (TP) content in peel methanolic extracts was determined using Folin-Ciocalteu (Folin C) colourimetric method as described by Makkar et al. (2007) and Fawole et al. (2012) with slight modifications. In triplicates, 450 µL of 50% methanol and 50 µL of extract were mixed with 1 N Folin-Ciocalteu reagent followed by 2% sodium carbonate. TP concentrations were determined spectrophotometically at 725 nm after 10 min of incubation in the dark. Gallic acid was used as a standard and results were expressed as mean ± S.E (milligrams) of Gallic acid equivalents (mg GAE g^{-1}DM).

2.5 Ferric reducing antioxidant power (FRAP) assay

Antioxidant capacity was determined by the FRAP assay of Benzie and Strain (1996) with slight modifications. The FRAP assay measures the ability of antioxidants in the sample to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to the blue colored ferrous form (Fe^{2+}) that absorbs light at 593 nm (Khanizadeh et al., 2008). In triplicates, 150 µLof the methanolic extract was mixed with 2850 µL of FRAP reagent (300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution, 20 mM ferric chloride) and incubated in the dark for 30 min. Absorbance at 593 nm was measured using a UV-vis spectrophotometer. Antioxidant capacity was expressed as mean ± S.E (micromoles) of trolox equivalents per milligram of dry matter (µM TE mg^{-1}DM).

2.6 DPPH radical scavenging activity

Peel extract was tested against a stable DPPH solution according to Wong et al. (2006) with some modifications. Briefly, a 0.1 mM solution of DPPH in methanol was prepared. The initial absorbance of DPPH in methanol was measured at 515 nm and did not change throughout the assay period. In triplicates, 15 µLof the extract was mixed with methanol (735 µL) and subsequently with DPPH solution (750 µL, 0.1 mM). The change in absorbance at 515 nm was measured after 30 min incubation at room temperature in the dark. Antioxidant capacity based on the DPPH free radical scavenging ability was expressed as mean ± S.E (millimolar) of ascorbic acid equivalent per milligram of dry matter (mM $AAEmg^{-1}DM$).

2.7 Lipid peroxidation

MDA was measured by the method described by Dhindsa et al. (1981) and Siboza et al. (2013) with slight modifications. Freeze dried and pulverised apple peel (0.1g) was homogenised with 10 mL of ice cold 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10000 rpm for 15 min at 4 ºC to precipitate particulates. A 1 mL aliquot of the supernatant was thoroughly mixed with 4 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was incubated at 95 °C for 30 min and thereafter quickly cooled in an ice bath. After centrifugation at 10000 for 15 min at 4 °C, the absorbance of the supernatant was read at 532 nm and corrected for nonspecific absorbance at 600 nm using UV-Visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). The concentration of MDA was calculated using an extinction coefficient (∈) of 155 mM^{-1} cm^{-1}.

3. Statistical analysis

Data obtained from the first season (2013) were subjected to correlation analysis using XLStat ver. 7.5.2 (Addinsoft, New York, USA) and solver in Microsoft Excel (Microsoft Office 2010, USA) was used to perform stepwise regression. Regression equations obtained were used to predict superficial scald incidence in the second season (2014).

4. Results and discussion

Results of the correlation analysis showed that all the measured fruit variables, aside of α-farnesene and DPPH, correlated significantly ($p<0.05$) with scald incidence, and consequently, α-farnesene and DPPH were excluded from the regression modelling (Table 1). Lipophilic antioxidants, as measured using DPPH assay in this study, play an important role in scald resistance (Barden and Bramlage, 1994). In fact, Zhang and Shu (2003) in their attempt to develop a prediction model for scald development argued that the ratio of α-farnesene to conjugated trienes (CTs) reflects the actual antioxidant levels of stored fruit. Lipophilic and hydrophilic antioxidant capacities as quantified by DPPH and FRAP assays, respectively, significantly changed with storage time and scald development. However, lipophilic antioxidants were not included in any of the prediction models. Zhang and Shu (2003) also found insignificant relationship between lipophilic antioxidant and scald incidence that would warrant its inclusion in the prediction model for 'Starking' and 'Ralls' apples. Interestingly, a recent attempt to develop a prediction model for 'Granny Smith' apples, Giné Bordonaba et al. (2013) also found peel antioxidant capacity to have low predictive power. Even though lipophilic antioxidant capacity significantly changed between storage regimes, its correlation to scald incidence was insignificant ($p =0.647$; refer to paper 5, fig 5). Lipophilic antioxidants can therefore not be used as a predictive marker of superficial scald development.

The accumulation of α-farnesene in apple peel during storage is widely accepted as the possible cause of superficial scald development. For instance, Lourens and Malherbe (1999) found α-farnesene to be one of the important predictive biomarkers for predicting superficial in 'Granny Smith' apples. In another study, Zhang and Shu (2003) found that the ratio of α-farnesene to conjugated trienes (CTs) maybe a reliable and easy predictor for scald development. In this study, although α-farnesene accumulation increased with storage time, it had insignificant correlation ($p =0.857$) with scald development. Based on this study, α-farnesene cannot be used as a reliable predictive biomarker as evidenced by its exclusion in the stepwise regression. This finding is comparable to Giné Bordonaba et al. (2013) who found α-farnesene not to be a suitable biomarker for scald prediction. In fact, Giné Bordonaba et al. (2013) reported that α-farnesene could only be used as the mid-term biomarker for scald incidence. This is attributed to its inability to predict scald after 2 months of cold storage. All the variables that significantly correlated with scald incidence were

analysed by stepwise regression to give the equations for scald prediction (Table 1). As shown in Table 1, three models were developed.

Model A

As expected, MHO levels increased with storage time and its values were highly correlated with scald incidence. Consequently, MHO was identified as a biomarker of scald incidence and gave the model A [$y = -0.01x^2 + 1.69x – 29.08$ (R^2= 0.98)] for scald prediction. The model appropriately predicted scald for both pre-optimally and optimally harvested fruit.

Model B

Ethylene production and lipid peroxidation correlation significantly with scald incidence ($p<0.05$) and together with scald were analysed by stepwise regression to give the equation of model B [y= 0.67 [Ethylene] + 8.67 [MDA] (R^2= 0.94)] for scald prediction.

Model C

Stepwise multiple regression indicated that the best combination of variables that could be used to predict superficial scald was MHO, antioxidants (FRAP), ascorbic acid and lipid peroxidation. This relationship gave a linear association with superficial scald development described by model C [y= 0.51 (MHO) – 0.93 (FRAP) + 2.90 (AsA) + 9.82 (MDA)] with good linear fit (R^2= 0.94).

Model application

The three prediction models were validated by comparing predicted values with actual values for each harvest maturity (Tables 2 and 3). The application of Model A generally gave poor predictions for both pre-optimally and optimally harvested fruit compared to the other models. This is shown by highly significant ($p<0.05$) t-test between predicted and actual scald incidence. Despite this limitation of Model A, it does suggest that MHO accumulation dynamics during the first 10 w of storage could be used as potential biomarker for scald prediction. The lower prediction accuracy of MHO alone on scald development may also be attributed to its reported crucial role in initiating superficial scald symptoms following the accumulaton generated from the oxidation of α-farnesene (Mir et al., 1999; Whitaker and Saftner, 2000).

The application of Model B (combination of ethylene production and lipid peroxidation) only gave a scald prediction for optimally harvested fruit. The inconsistency of model B to predict scald in different maturities indicates that pre-optimally and optimally harvested fruit respond differently to cold stress. The inclusion of MDA in model B corroborates with previous finding observed in 'Gala' and 'Golden Smoothee' apples (Vilaplana et al., 2006; Shao et al, 2012) that MDA could be used as an indicator of the development of postharvest disorders. In contrast, Giné Bordonaba et al. (2013) reported that MDA cannot be used as a predictive biomarker for scald. This was attributed to the unexpected reduction of MDA during storage. In this present study, MDA progressively increased during storage (Chapter 5, Fig. 3).

The combination of variables in Model C was identified as the best predictor of scald development both pre-optimally and optimally harvested fruit. The inclusion of lipophilic antioxidants in model C highlights the important role of fruit antioxidant status in resisting superficial scald. High antioxidant concentrations could inhibit the oxidation of α-farnesene to MHO and subsequently result to no scald development.

Model validation

All the three models were validated in a subsequent year with fruit from pre-optimal and optimal maturity stored in air. Of the three models, model C appropriately described the influence of MHO accumulation, antioxidants and ascorbic acid concentration, and lipid peroxidation on scald development as evidenced by high R^2 for both pre-optimally (R^2 = 0.993) and optimally (R^2 = 0.968) harvested fruit. The scatter plot (Fig. 1C and 2C) showed a good relationship between experimental and predicted scald incidence. Furthermore, model A showed experimental and predicted values for scald incidence to be comparable. In terms of predictive ability of the three regression models, model C had highest predictive ability and reliability followed by model A and model B.

Practical implications of models

A prediction model should be reliable and an easy predictor of scald development regardless of fruit maturity. Although the multiple regression model C appears to be more reliable and accurate in predicting scald development, it is certainly not an easy scald predictor to use, especially from an industry perspective. In model C, four variables should be

measured before scald development could be predicted. Each of the variables has a specific protocol for measurement. Model C could therefore be categorized as labour intensive and not practical enough to be used by the apple industry. However, this model could be practical if technological interventions could be used to develop quick non-destructive techniques for measuring the scald predictor variables. Although model A, which only focuses on MHO dynamics during storage, is not reliable enough to predict scald, it would be a handy and easy predictor of scald development. Further studies are warranted to test the models extensively using other fruit from different growing areas across production seasons.

5. Conclusion

Overall, effective predictive variables for superficial scald development have been identified. This study has shown that the combination of MHO, hydrophilic antioxidant capacity, ascorbic acid concentration and lipid peroxidation could be applied to predict scald development in 'Granny Smith' apples during cold storage. However, although it could be commended for its accuracy, this predictor is not easy to employ due to many variables that should be measured using elaborate wet chemistry. Although Model A which only focuses on MHO dynamics during storage is not reliable, it would be a handy and easy predictor for scald development. Model C reported in this study offers a new tool to predict the storage life of 'Granny Smith' apples to minimise scald development and further extensive testing of the model is warranted to demonstrate the industrial application.

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Table 1

Prediction models for superficial scald development on 'Granny Smith' apples

Model	Equation to predict scald incidence (%)	Regression (R^2)
A	Y= -0.01[MHO]^2 + 1.69[MHO] – 29.08	0.98
B	Y= 0.67 [Ethy] + 8.67 [MDA]	0.94
C	Y= 0.51 [MHO] – 0.93 [FRAP] + 2.90 [AsA] + 9.82 [MDA]	0.94

Table 2

Predicted scald incidence and accuracy of prediction from three models, and actual scald incidence of pre-optimally harvested 'Granny Smith' apples

Model A			Model B			Model C			Actual %Scald
Predicted	**Residuals**	**P-value**	**Predicted**	**Residuals**	**P-value**	**Predicted**	**Residuals**	**P-value**	
-2.25	2.25	<0.001	18.44	-18.44	<0.001	5.35	-5.35	<0.001	0.00
39.05	-3.39	**0.438**	60.8	-25.15	<0.001	35.23	0.44	**0.448**	35.67
85.98	-1.80	0.006	68.97	15.21	<0.001	81.79	2.39	0.032	84.18
86.91	11.43	0.003	90.22	8.11	0.045	100.00	1.67	**0.443**	98.33
84.31	15.10	0.003	92.63	6.77	0.005	100.00	0.64	**0.107**	99.40

Table 3

Predicted scald incidence and accuracy of prediction from three models, and actual scald incidence of optimally harvested 'Granny Smith' apples

Model A			Model B			Model C			Actual % Scald
Predicted	**Residuals**	**P-value**	**Predicted**	**Residuals**	**P-value**	**Predicted**	**Residuals**	**P-value**	
-3.75	3.75	**0.078**	18.15	-18.15	<0.001	-4.90	4.90	0.017	0.00
42.45	3.97	**0.169**	53.13	-6.71	**0.062**	39.03	7.39	**0.104**	46.42
83.09	1.91	0.012	79.41	5.59	0.032	85.31	-0.31	**0.051**	85.00
84.04	13.59	<0.001	98.10	-0.47	**0.389**	99.99	-2.36	<0.001	97.63
83.25	16.61	<0.001	97.42	2.44	**0.060**	93.13	6.73	**0.054**	99.86

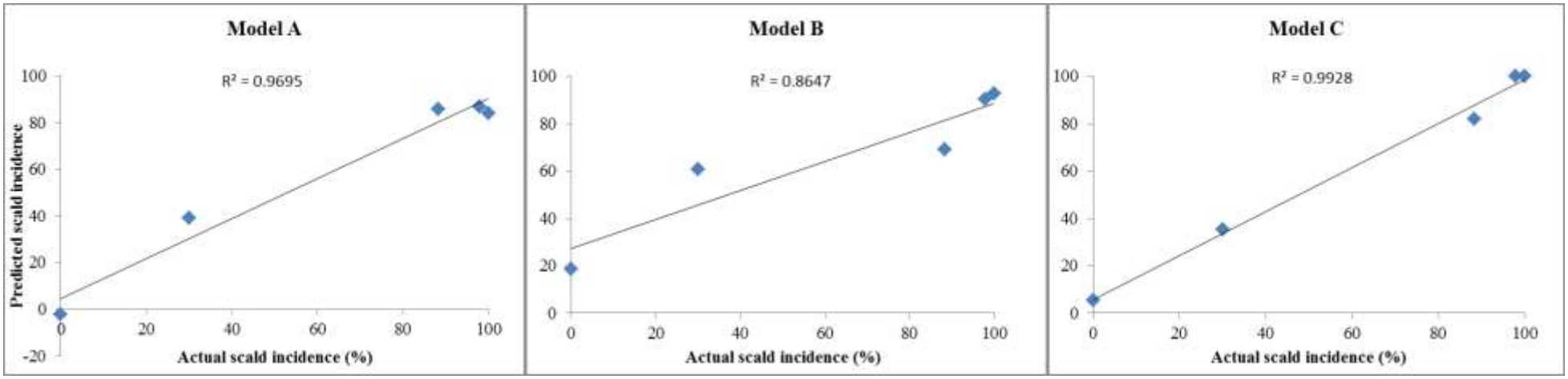

Fig. 1. Relationship between the actual and predicted scald incident values of 'Granny Smith' apples harvested at pre-optimal maturity.

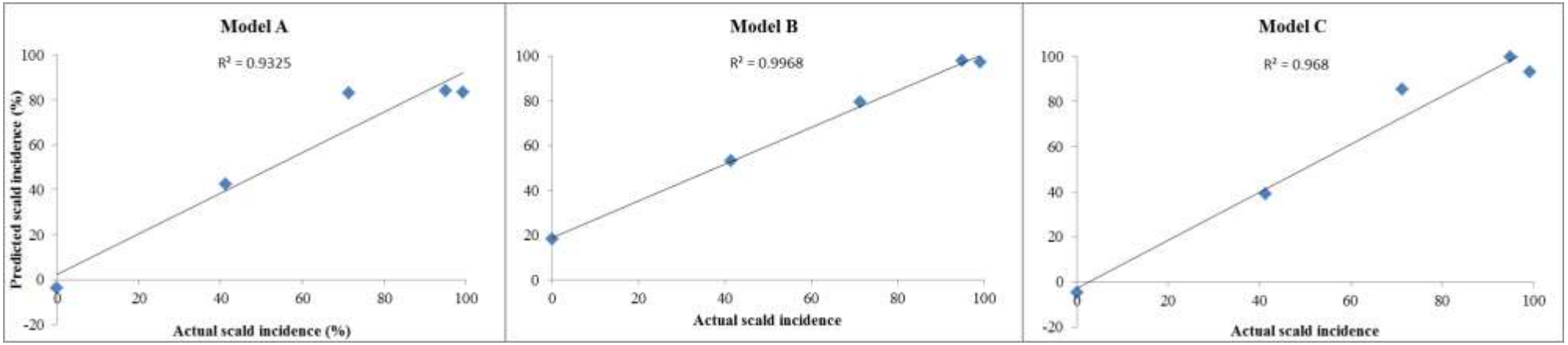

Fig. 2. Relationship between the actual and predicted scald incident values of 'Granny Smith' apples harvested at optimal maturity.

GENERAL DISCUSSION AND CONCLUSION

1. Introduction

Apples are one of the most widely consumed and important fruit in the world. Like other fruits, the consumer demand of apples is highly influenced by external quality. The long cold storage duration of apples often reduce fruit appearance and subsequently lead to low consumer demand and low net-profit for producers. In 'Granny Smith' apples, superficial scald is the main physiological disorders reducing the quality of stored fruit. Postharvest chemical treatments such as 1-methlycyclopropene (1-MCP) and diphenylamine (DPA) are highly effective in controlling this disorder. However, chemical treatments generally pose health concerns to consumers. Consequently, the international market share for chemically free fruit is rapidly growing.

These recent developments have forced the South African Apples Fruit Industry to reform if it has to stay competitive in international markets. Therefore, the overall aims of this study was to (a) investigate the potential of the recent discovered technology, dynamic controlled atmosphere, to control superficial during long cold storage of 'Granny Smith' apples, (b) to investigate the mechanism of action used by DCA to control superficial scald, should it be effective. The specific objectives of this study were to (a) Assess effects of DCA on scald and biochemical precursors in fruit at different maturities; (b) Determine critical application period for DCA to inhibit scald; (c) Investigate the influence of intermittent breaks on DCA effectiveness; and (d) identify predictive variables that could be used to predict scald development. Better understanding of mechanism of action employed by DCA to control scald development will assist in optimising the DCA technology. The outputs of this study will provide new insights of superficial scald control; new storage protocols for 'Granny Smith' apples will also be developed, moreover, sustainability and competiveness of apples fruit industry in South Africa.

Accordingly, this dissertation was structured into the following eight papers

- Paper 1: Superficial scald in apples: Biochemistry, physiology, control and modelling-A review
- Paper 2: Antioxidants contents and phytochemical properties of apples (cv. 'Granny Smith') at different harvest times

- Paper 3: Classification of 'Granny Smith' apples with different levels of superficial scald severity based on metabolomics and discriminant analysis
- Paper 4: Minimum exposure period for dynamic controlled atmospheres to control superficial scald in 'Granny Smith' apples for long distance supply chains
- Paper 5: Effect of dynamic controlled atmospheres on reactive oxygen species, antioxidant capacity and phytochemical properties in 'Granny Smith' apples
- Paper 6: The efficacy of repeated application of dynamic controlled atmospheres of scald potential 'Granny Smith' apples
- Paper 7: Effect of dynamic controlled atmospheres on volatile compound production in 'Granny Smith' apples
- Paper 8: Identification of effective predictive variables for superficial scald in 'Granny Smith' apples

2. General discussion

Superficial scald in apples: Biochemistry, physiology, control and modelling- a review

This section introduced the dissertation and reviewed literature on storage and superficial scald development of apples. The objective of **Paper 1** was to discuss current developments on superficial scald research; recent technological interventions on scald development are also discussed. Literature evidence showed that superficial scald development in apples is linked to accumulation α-farnesene and its oxidation products, 6-methyl-5-hepten-2-one (MHO) and conjugated trienols (CTols) (Mir et al., 1999; Wang and Dilley, 2000; Lurie and Watkins, 2012; Busatto et al., 2014; Farneti et al., 2015). Moreover, effective chemical postharvest treatments such 1-MCP and DPA reduce the accumulation of these volatile compounds (Whitaker, 2000; Isidoro and Almeida, 2006; Jung and Watkins, 2008). Furthermore, scald development is also linked to lipid peroxidation during storage (Thomai et al., 1998; Vilaplana et al., 2006; Lu et al., 2011; Shabban-Amin et al., 2011; Lu et al., 2014). Antioxidant capacity and certain phytochemicals are also involved in scald resistance in stored fruit (Ju and Curry, 2000; Arquiza et al., 2005; Abbasi et al., 2008).

Literature evidence has shown that the genetic component coupled with enzyme activity is another predominant in intrinsic factor influencing superficial scald etiology in apples. The synthesis and oxidation of α-farnesene, which plays a critical role in scald development, is closely linked to α-farnesene synthase (*AFS1*), an enzyme which converts

farnesyl diphosphate to α-farnesene (Rupasinghe et al., 2000; Whitaker, 2004; Sabban-Amin et al., 2011; Busatto et al., 2014). In fact, genetic variation during cold storage has been reported. The expression of Pc*AFS1* is commonly known to precede α-farnesene and CTols accumulation (Gapper et al., 2006). Interestingly, both Pc*AFS1* expression and α-farnesene content are significantly reduced by scald inhibiting treatments such as1-MCP application (Gapper et al., 2006; Pechous et al., 2005). The browning in scalded fruit has specifically been correlated to chlorogenic acid accumulation which is activated by *MdPAL, MdPPO* and *MdC3H* gene (Busatto et al., 2014). Interestingly, *MdPPO* which has previously been correlated to scald development (Shabban-Amin et al., 2011) is directly responsible for chlorogenic acid oxidation (Busatto et al., 2014). Regardless of these advances in understanding the genetics of scald etiology, scald resistant 'Granny Smith' cultivar has not yet been developed. Consequently, recent research efforts have been concerted on finding non-chemical methods of controlling superficial scald.

Low oxygen storage for inhibiting scald development has gained momentum in the past decade. Array of low oxygen storage technologies have been developed which include initial low oxygen stress (ILOS; Ghahramani and Scott, 1998; Pesis et al., 2010; Sabban-Amin et al., 2011; Pesis et al., 2010; Pesis et al., 2014), ultra low oxygen (ULO; Lau, 1990; Lau, 1997) and more recently, dynamic controlled atmosphere (DCA; Yearsley et al., 2003; Weber et al., 2015; Wright et al., 2015; Tran et al., 2015). Currently, literature relating to the efficacy of low oxygen storage, particularly DCA, to control superficial scald is limited. This is not surprising given that the DCA is quiet a new technology in the apple industry. Therefore, DCA technology and its application to 'Granny Smith' apples should be thoroughly studied to develop storage protocols that will ensure that consumers get good quality, tasty and flavourful 'Granny Smith' apples.

Antioxidants contents and phytochemical properties of apples (cv. 'Granny Smith') at different harvest times

It is commonly known that advanced fruit maturity reduces the propensity of postharvest physiological disorders during cold storage (Fan et al. 1999). In contrast, pre-optimal maturity may not fully ripen after storage and are highly susceptible to physiological disorders and bruising (Saevels et al. 2003; Opara, 2007). The major setback of late harvested fruit is softness and greasiness after storage (Fan et al. 1999). The involvement of ethylene gas in the aspects of ripening is well known (Fan et al. 1999; Zanella, 2003). Although

ethylene manipulation is considered a 'standard' practise in maintaining the quality of stored produce, other physiological aspects play a prominent role in postharvest performance particularly in fruit from different maturities. The objective of **Paper 2** was to assess the antioxidants contents and phytochemical properties of apples (cv. 'Granny Smith') harvested at 7 d before and at optimal commercial harvest. This study provides useful information on physiological and biochemical variables distinguishing pre-optimally and optimally harvested apples. This knowledge might be useful in optimising postharvest cold and supply chain management of fruit.

The study showed significant biochemical differences between pre-optimally and optimally harvested fruit (**Paper 2**). Ascorbic acid content and total antioxidant capacity (as measured by FRAP and DPPH) were significantly higher in optimally (H2) compared to pre-optimally harvested fruit (H1). Interestingly, total phenolics and phenolic compounds including catechin, epicatechin and quercetin were significantly lower in H2 compared to H1 fruit. Moreover, correlation was found between total phenolics and catechin (r^2=0.658) and between catechin and epicatechin (r^2=0.687) phenolics. The reduced total phenolics and phenolics compounds in H2 could be linked to polyphenol oxidase which declines during ripening process (Kim et al. 2011). This study suggests that the high postharvest potential of optimally harvest apples could be linked to high antioxidant potential in this fruit. Moreover, postharvest technologies should be designed in a manner that minimizes the loss of these important antioxidants.

Classification of 'Granny Smith' apples with different levels of superficial scald severity based on metabolomics and discriminant analysis

Metabolomic events leading to the development of superficial scald in apples have been studied. However, the relationship between scald severity and accumulation of metabolites linked to scald development remains unclear. The study reported in **Paper 3** was aimed towards investigating the relationship between scald severity and metabolomic changes. The findings reported in this study showed that ethylene production rate, α-farnesene and MHO contents and ROS intensity increased with scald severity but declined in severely scalded fruit. Moreover, lipid peroxidation in the peel increased linearly (R=0.891) with scald severity. With the use of discriminant analysis, the investigated scald severity indexes were clustered into five groups. The groups were based on the amount of metabolites accumulated. The stepwise model indicated that three attributes (ROS, ethylene production

and MDA) contributed significantly ($R^2 \geq 0.5$) to the separation of the five scald severity indexes, with ROS having the highest contribution (partial $R^2 = 0.961$; $p < 0.0001$), followed by ethylene ($R^2 = 0.718$; $p < 0.0001$) and MDA ($R^2 = 0.578$; $p < 0.0001$). This study demonstrated that ROS accumulation which leads to loss of membrane integrity strongly corresponds to scald severity levels. These findings evidently suggest that scald resistance of 'Granny Smith' apples might be enhanced by improving fruit ability to metabolise ROS during storage.

Minimum exposure period for dynamic controlled atmospheres to control superficial scald in 'Granny Smith' apples

Recent research on low oxygen storage has demonstrated that DCA is effective in inhibiting scald development in apples (Yearsley et al., 2003; Weber et al., 2015; Wright et al., 2015; Tran et al., 2015). The objectives of **Paper 4** were aimed at assessing the potential of DCA to control superficial scald in pre-optimally and optimally harvested 'Granny Smith' apples; the critical minimum period for DCA to control scald was also investigated. This study was conducted from a practical point of view that, unlike other markets such as Italy (a major user of DCA technology for storage of apples) that are in close proximity to their consumers, South Africa has distant markets which require shipping of the produce at low temperatures for 6 to 10 w. Indeed, results obtained showed that DCA is effective in controlling scald development regardless of fruit maturity. Shipping the fruit at -0.5 °C for 6 w (in RA) after DCA treatment had no negative effect on fruit quality. However, shipping fruit for 10 w at RA after DCA treatment increased the risk of superficial scald development. This finding has demonstrated that DCA does not necessarily prevent scald development, but delays the symptoms.

The overall quality of DCA stored fruit was good. Moreover, the evolution of scald associated metabolites (α-farnesene, MHO and ethylene) was evidently suppressed in DCA. Using Pearson correlation analysis, scald incidence and MHO showed a strong relationship in fruit stored only in RA ($r = 0.863$) and a weaker relationship in DCA stored fruit ($r = 0.365$). Now that it's been established that shipping fruit for 10 w after DCA treatment leads to scald development, the next step in this study would be to investigate whether fruit shipped for 7, 8 or 9 w could be scald free. This is attributed to the fact that one additional week for fruit shipment without scald development could be of enormous value to the South African Fruit Industry. Owing to this reason, it is imperative that further studies be conducted to investigate this possibility.

Effect of dynamic controlled atmospheres on reactive oxygen species, antioxidant capacity and phytochemical properties in 'Granny Smith' apples

Studies on superficial scald have largely focused on the effect of postharvest treatments on scald associated metabolomic attributes such as ethylene, α-farnesene, CTs and MHO. The effect of DCA on antioxidant attributes during storage has not yet been studied. The study reported in **Paper 5** was aimed at assessing the evolution of antioxidant capacity, phytochemical contents and reactive oxygen species in DCA stored apples harvested at pre-optimal and optimal maturity. In this study, we hypothesized that DCA inhibits scald by retarding the loss of antioxidants and phytochemical properties of the fruit tissue, thereby reducing ROS accumulation and lipid peroxidation.

Fruit stored in DCA exhibited high antioxidant, ascorbic acid and total phenolics concentration. This coincided with zero scald incidence. With the use of principal component analysis, two groups that could easily be identified as DCA and RA stored fruit were clustered. Interestingly, lipid peroxidation and ROS associated with RA stored fruit whilst antioxidant capacity, ascorbic acid and total phenolics strongly associated with DCA stored fruit. This is supported by the work of Rao et al. (1998), Zubini et al. (2007), Shabban-Amin et al. (2011) and Lu et al. (2014), who demonstrated a strong relationship between poststorage fruit quality and ROS levels in apples. It can be deduced that the high fruit quality in DCA is due to higher antioxidant pool in this fruit. It is noteworthy that unlike fruit harvested at pre-optimal (which was highly prone to scald development), fruit harvested at optimal maturity had superior antioxidant pool. It can be concluded that the resistance to physiological disorders and high storage potential of fruit harvested at optimal maturity is linked to high antioxidant pool. The higher antioxidant pool evidently retards the development of ROS, accumulation of toxic volatile compounds and consequently reduce membrane deterioration, a metabolic activity that determines fruit quality and its market value.

The efficacy of repeated application of dynamic controlled atmospheres of high scald potential 'Granny Smith' apples

The research study reported in **Paper 6** was aimed at investigating the efficacy of repeated application of DCA on scald development. This study was conducted from a practical point of view that, an unanticipated demand of fruit and the need to remove orchard

lots from cold storage requires the opening and resealing of DCA chambers after packing. During packing, the storage condition is the normal refrigerated air as opposed to low oxygen levels under DCA. It remained unknown whether repeating DCA application following fruit packing at RA is effective in controlling scald. Indeed, the results obtained showed that the repeating DCA application is effective in controlling scald. However, the development of 1% and 2% scald during the season 1 and season 2, respectively, is of great concern to fruit producers. This is due to the zero tolerance to scald for the export markets. It could be deduced that the 14 d packing period in RA is too long. Future studies should establish whether the reduced packing period from 14 d to 8-10 d could be beneficial to DCA efficacy and fruit quality.

Using principal component analysis, metabolic changes and their relationship was visualized and two clusters that could easily be identified as RA and repeated DCA were observed. Interestingly, high firmness, ground colour and titratable acidity were strongly associated with fruit repeatedly stored in DCA. On the other hand, fruit constantly stored in RA was characterized by high ethylene production, α-farnesene and MHO accumulation which had a strong and positive correlation with scald incidence. These findings could provide a very useful storage protocol to ensure the fruit does not develop scald during storage, shipment and shelf-life. This is critical in maintaining the competitiveness of the South African apple industry which heavily relies on international markets.

Effect of dynamic controlled atmospheres on volatile compound production in 'Granny Smith' apples

Low oxygen levels during storage have a significant influence on aroma volatiles of apples (Mattheis et al., 1998; Raffo et al., 2009; Echeverria et al., 2004). The objective of **Paper 7** was to compare the aroma volatile profile of 'Granny Smith' apples of DCA storage with that stored in normal refrigerated air. A total of 21 aroma volatiles were detected in the headspace of the fruit juice. The results showed low number of volatiles in DCA stored fruit compared to those stored under RA conditions. The low number of volatiles in DCA was not surprising as it has previously been reported (Raffo et al., 2009).

Ethyl-2-methylbutyrate, ethyl hexanoate and hexyl acetate were identified as the major contributors to the characteristic flavour of 'Granny Smith' apples. Their level was higher throughout the storage duration. It was noteworthy that ethanol production was higher

in DCA stored fruit compared to those stored in RA. It is well known that ethanol is the precursor of esters such as ethyl 2-methyl butanoate that have low organoleptic threshold (Flath et al., 1967; Lara et al., 2006). However, there were no ethanol derived esters detected in this study. Additionally, no acetaldehyde was detected during storage, meaning that ethanol was not oxidised. It could be deduced that the high ethanol accumulation in DCA is linked to the mechanism in which low oxygen levels inhibit scald development (Ghahramani and Scott, 1998; Wang and Dilley, 2000). Future studies should investigate this possibility as it might be instrumental in optimising low oxygen storage technologies. Real-time and non-destructive techniques for the investigation of aroma volatiles in apples should also be investigated. Literature review showed that this was the first work on the evolution of aroma volatiles of 'Granny Smith' apples stored in DCA. Previous studies on the effect of DCA on aroma volatiles only focused on 'Gala', 'Pinova' and 'Fuji' apples (Mattheis et al., 1998; Raffo et al., 2009; Echeverria et al., 2004).

Identification of effective predictive variables for superficial scald development in 'Granny Smith' apples

Quality management at postharvest and during the distribution of fruit is highly important (Opara, 2009). It is not enough to develop postharvest technologies for maintaining quality. The interaction of the complex factors including preharvest cultural practices and postharvest management affect metabolic activities and consequently fruit quality. Recently, prediction models have shown their ability create allometric relations to predict quality traits of the stored produce (Nicolaï et al., 2008; Magwaza et al., 2013). Efforts have been made to develop prediction models for scald development. However, in South Africa we do not have a prediction model for scald development. Moreover, potential scald predictive variables have not been identified. Therefore, the objective of **Paper 8** was to identify variables with good predictive power that could be used to predict superficial scald development of 'Granny Smith' apples. Indeed, the results showed ethylene, MHO and lipid peroxidation to be positively correlated with scald development. On the other hand, a negative correlation between ascorbic acid, antioxidant capacity (as measured by FRAP) and scald incidence was noticed.

The relationship between MHO and scald development nicely fitted the quadratic equation [% scald incidence (A) = $-0.01x^2 + 1.69x - 29.08$; $R^2 = 0.98$]. Another model [% scald incidence (B) = 0.67 (Ethylene) + 8.67 (MDA); $R^2 = 0.94$] also gave a good scald

prediction. Across all variables, the stepwise multiple regression identified MHO, antioxidant capacity (FRAP), ascorbic acid concentration and lipid peroxidation to be the best combination of predictors for scald development (scald incidence % (C) = 0.51 (MHO) – 0.93 (FRAP) + 2.90 (AsA) + 9.82 (MDA); R^2= 0.94]. After validation, the combination of factors in model C proved to be the most reliable model. However, the limited practical application due to many factors that should be measured is a major concern for model C. With technological interventions that could non-destructively measure scald associated metabolites, model C could be used. The recent development and validation of 'index of absorbance difference' (IAD), as measured with a DA-Meter, a portable device based on NIR, to measure fruit maturity and predict scald development (Farneti et al., 2015) points to a brighter future for postharvest quality measurement and control, including scald. In fact, this demonstrate that non-destructive techniques for quantifying scald-associated metabolites might be the epitome of scald research in the next couple of years. Designing technologies that can measure immediate-and-real-time physiological and microstructural dynamics during storage is highly imperative.

The identified variables in this study should be used to develop a model that could predict scald before it appears; moreover, non-destructive techniques should also be developed for easy and handy quantification of these variables. Furthermore, future research should also investigate the potential of DA-Meter to predict scald development in the South African apple industry.

3. General conclusion and future prospects

In conclusion, this study has presented findings on the potential of dynamic controlled atmospheres to control superficial scald in 'Granny Smith' apples. This dissertation addresses topics that are of importance and relevance to the South African and global apple industry. Furthermore, it shows how DCA, as a non-chemical postharvest treatment, could be used to achieve the prospects of a continuing highly competitive South African apple industry that is faced with the challenge of finding alternative effective management strategies for scald and similar postharvest disorders. In addition, it elucidates the mechanism of action used by DCA to retard scald incidence. However, this study did not establish whether the shelf-life of DCA stored fruit is equal to flavour life. Moreover, the influence of delayed DCA application on scald control was not evaluated. Currently, there is no research data available on the flavour and organoleptic properties of DCA stored 'Granny Smith' apples. The scope of DCA

research should be extended to other cultivars, moreover, the potential of DCA to control other physiological disorders should be examined. Reseach on superficial scald remains expensive and time consuming, concerted reseach efforts should be focused on developing techniques that can non-destructively measure the real-time physiological, biochemical and microstructural changes during storage. Such approach will lead to significant reduction in chemical usage and overall research costs.

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